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BIOLOGIC DECAY RATES OF CHLORIDE IN NORMAL AND DISEASED MAN, DETERMINED WITH LONG-LIFE RADIOCHLORINE,* Cl^{36}

C. T. Ray, M.D., G. E. Burch, M.D., and S. A. Threefoot, M.D.
New Orleans, La.

AVAILABILITY of long-life radiochlorine, Cl^{36} ($t_{1/2} \simeq 4.4 \times 10^5$ years), has made possible direct measurement of certain aspects of chloride metabolism in man heretofore impossible with either ordinary chloride, Cl^{35}, or the short-life radioactive isotope, Cl^{38} ($t_{1/2} \simeq 37$ minutes). Among the aspects of chloride metabolism which may be studied only with Cl^{36}, for example, are the time required for the administered radiochlorine to reach equilibrium of distribution in the various chloride compartments of the body, rate of turnover of total body chloride by various excretory routes, measurement of total chloride mass in the body of an intact man, influence of various physiologic phenomena upon turnover rates of chloride, and influence of chloride intake and other factors upon the size of exchangeable chloride spaces.

Previous experiments with Na^{22} [1] and with $Hg^{203, 205}$ [2] showed striking differences in biologic decay rates for man suffering from chronic congestive heart failure and for control subjects. Furthermore, it is known that chronic congestive heart failure is associated with disturbances in metabolism of electrolytes and water, and with the long-life radiochlorine it was possible to observe certain aspects of differences in chloride metabolism in these patients and in normal man. Properly designed experiments also make it possible to determine the biologic decay rates or turnover rates of the element for the entire body of man, as well as the total body mass of chloride under selected dietary conditions and the influence of other selected physiologic and pharmacologic factors. Such observations are significant for a better understanding of the edema of congestive heart failure and the associated abnormal water and salt metabolism as well as certain general aspects of salt and water metabolism. These studies also throw light upon the problem of radiobiologic injury in both peacetime and military use of radioelements involving radiochlorine. This report is concerned primarily with observations of the biologic decay periods in man suffering with chronic congestive heart failure and in control subjects.

METHODS AND MATERIALS

The nature and duration of these experiments permitted adequate study of only three patients simultaneously. Accordingly, two series of essentially similar experiments were conducted on a total of six female patients from the Charity Hospital. Each series of

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actual observations lasted from two to three months. The patients were placed in special metabolic beds and were under constant observation by an attendant whose duties were to control procedures, record dietary, fluid, and drug intake, and collect all excreta. In each group of three subjects under simultaneous observation, one was free from cardiovascular disease, one was a patient with mild to moderately severe congestive heart failure, and the third had severe failure. Thus, two patients of each type were observed. All were free from renal disease. Since these patients were studied simultaneously, the control subjects and those with congestive heart failure were subjected to the same uncontrollable variables, as, for example, climate. The pertinent clinical data are presented in Table I.

TABLE I. CLINICAL DATA FOR SIX HUMAN FEMALE SUBJECTS WHO RECEIVED INJECTIONS OF Cl^{36}

SUBJECT NO.	DOSE	METHOD OF ADM.	AGE (YR.)	WEIGHT (LBS.)	DIAGNOSIS	TREATMENT
A. Normal or Control						
501	10,000,000	Rapid I.V.	31	104.0	Thyrotoxicosis	Regulated with propylthiouracil
504	10,200,000	Rapid I.V.	44	110.75	Tuberculous adenitis	Treated with streptomycin
B. Congestive Heart Failure						
502	10,000,000	Rapid I.V.	62	90	Diabetes, hypertensive cardiovascular disease	
505	12,500,000	Rapid I.V.	50	142.5	Arteriosclerotic heart disease	
503	10,000,000	Rapid I.V.	59	148.0	Died; severe congestive heart failure, arteriosclerotic heart disease	
506	10,000,000	Rapid I.V.	54	101.5	Died; severe congestive heart failure, hypertensive and arteriosclerotic heart disease, pulmonary emboli with infarction, aortic insufficiency	

A period of five to seven days was allowed for the subjects to become stabilized on a control diet. The subjects were moved from Charity Hospital to our laboratory for injection of radiochlorine and observation of the initial period of rapid transfer of the isotope out of the blood plasma. The radiochlorine, Cl^{36}, was injected intravenously in a dose of about 10,000,000 counts per minute, or approximately 50 μc., in a volume of 10 to 15 ml. of normal saline. This required from eight to thirty seconds. Simultaneously with the beginning of injection into one median basilic vein, blood was drawn under oil from a vein of the opposite arm. Collection of blood was made every five to ten seconds for the first three minutes, every fifteen to thirty seconds for the next two minutes, and then every minute until ten minutes had elapsed. Thereafter samples were drawn at three- to five-minute intervals until thirty minutes had elapsed, and then at ten- to fifteen-minute intervals until two hours had elapsed following injection. Simultaneously urine was collected by means of an indwelling bladder catheter. Red cells were separated from serum by centrifugation, and the radioactivity of the samples of serum and urine was determined by Geiger counters by the method previously described.[3]

Following completion of this initial phase of observation designed to measure the rate of transfer of chloride from serum, including its appearance in the urine, the subjects were returned to the ward for the prolonged period of observation. During this time the diets

were controlled and all medication and fluid intake were measured.* A sample of blood was collected at approximately the same time each morning. Each time urine was voided, it was collected in separate bottles to permit determination of individual urinary values as well as twenty-four hour values. Stools, vomitus, sputum, and pleural and ascitic fluids were also collected and assayed, total volume being recorded in all instances. From time to time dietary intake of chloride and other electrolytes was varied by administration of tablets or capsules of ammonium chloride, sodium chloride, sodium bicarbonate, potassium citrate, or sodium bromide. The quantities and time of administration of these as well as such drugs as DOCA (desoxycorticosterone acetate), cortisone, Mercuhydrin, and Thiomerin will be indicated in the results.

Simultaneous measurements of stable chloride, Cl^{35}, sodium, and potassium were made. Chloride was determined by the method of Schales and Schales,[4] and sodium and potassium by flame photometry, suitable corrections having been made for mutual excitation of the Na and K ions.

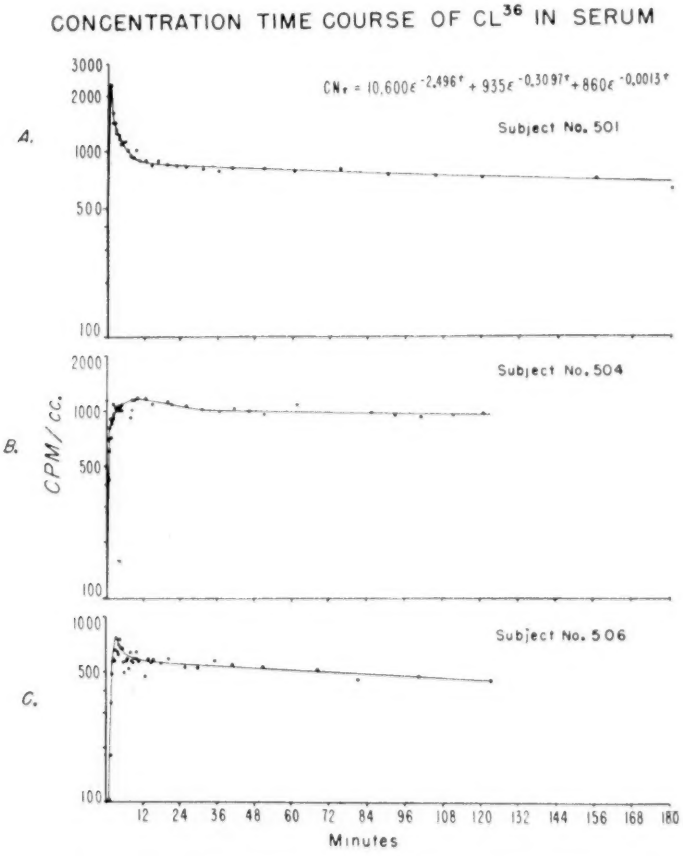

Fig. 1.—Early concentration-time course of Cl^{36} in blood serum. Consult text for details.

RESULTS

Early Concentration-time Course of Cl^{36} in the Blood Serum.—From the sera collected during the early phase of the experiments immediately following

*The cooperation in these studies of Sister Euphemia and Misses Gloria Kicklighter and Jeanne Reid of the Dietary Department of Charity Hospital is greatly appreciated.

injection, it was possible to plot curves of concentration-time course for the six subjects studied. Essentially two types of curves were obtained. Fig. 1, *A* shows the curve obtained for control Subject No. 501 in which there was a rapid accession followed by a less rapid regression and which resembles the mean curve obtained for dogs.[5] The second type of curve (Fig. 1, *B*) was obtained for control Subject No. 504, in which there was a rapid accession to a level which failed to regress rapidly but rather tended to decline in concentration at a rate comparable to the slowest rate seen in the curve for Subject No. 501. The curves for the other four subjects were similar to that shown in Fig. 1, *B*. Fig. 1, *C* is presented to show the regression curve of one subject with severe heart failure. Of the curves exemplified by that shown in Fig. 1, *B*, the accession to essentially the maximal level was completed definitely more rapidly by the control subjects (twenty seconds) than by the four subjects with heart failure (100, 120, 210, and 300 seconds).

The curves shown in Figs. 1, *B* and 1, *C* do not permit an estimation of rates of transfer of chloride across the blood vessel wall by the method described by Merrell and associates,[6] whereas this is possible from Fig. 1, *A*, according to the analysis described by Merrell and that employed by us for sodium,[7] mercury,[8] and chloride.[5] The regression of concentration with time was found to follow the mathematical expression:

$$CN_t = 10600e^{-2.496t} + 935e^{-0.3097t} + 860e^{-0.0013t},$$

where CN_t is the concentration of Cl^{36} in the serum at any time t, t represents time in minutes after injection of the tracer, and e is the base of the natural logarithm.

Comment.—The causes for the two different types of curves of concentration-time course shown in Fig. 1 are unknown; several explanations have been offered in previous publications.[7, 8] For example, the injections were made relatively slowly (eight to thirty seconds) and in fairly large volumes of fluid (10 to 15 ml.). With slow injections the rapidly diffusing chloride ions disappear from the serum so quickly that maximum concentration of the tracer is not much higher than the level of equilibrium (Fig. 1, *B*). The concentration curve shown in Fig. 1, *A* is of the usual multiple exponential decay rates previously discussed.[8] It is evident that there are multiple rates of regression of concentration with time. The first or most rapid rate, with the exponential value of –2.49, contains a mixing element of unknown value which renders impossible the precise interpretation of the transfer factor out of the serum.

TABLE II. DISTRIBUTION AND TURNOVER OF CHLORIDE IN BLOOD SERUM AND EXTRASERAL COMPARTMENTS FOR SUBJECT NO. 501

q	= Proportion of total chloride not in serum	0.93
1-q	= Proportion of total chloride in serum	0.07
R_s	= Proportion of seral chloride leaving serum per minute	2.16
R_e	= Proportion of nonseral chloride returning to serum per minute	0.16
$R_s(1-q) = R_e(q)$	= Proportion of total body chloride moving in or out of serum per minute	0.15
$R_s(1-q) + R_e(q)$	= Proportion of total body chloride moving in and out of serum per minute	0.3

That mixing may have contributed considerably to the first exponential decline is evident from results of calculations of the serum and extraseral partitioning of Cl^{36}. The calculations (Table II) resulted in a value for serum of about 7 per cent of total body chloride, a value which is apparently too low. However, the nature of the various dynamic chloride compartments of the serum and the respective rates of exchange between the subcompartments contributing to each are unknown. These unknown factors preclude any definite conclusions. Nevertheless, it is safe to state that the curves of concentration-time course for Cl^{36} are similar to those for Na^{22} [1] and Na^{24} [7] and that Cl^{36} is transferred rapidly in and out of serum.

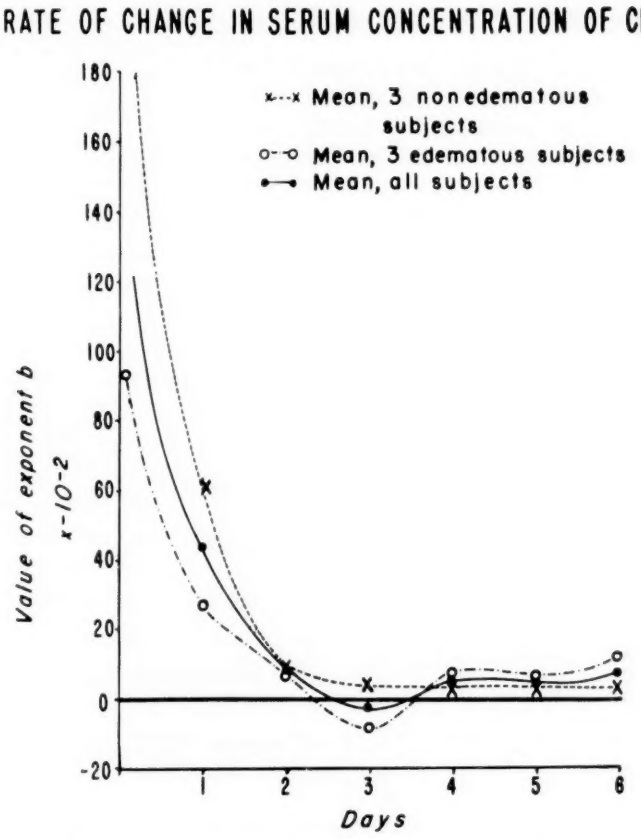

Fig. 2.—Rate of change in serum concentration of Cl^{36}. The values of the ordinate represent the values of exponent b, an expression of the slope of the concentration-time course curves.

EQUILIBRIUM OF DISTRIBUTION

With an isotope of long physical half-life, such as Cl^{36}, it is possible to estimate the time at which equilibrium of distribution of the tracer is reached. Certain criteria are necessary to establish the fact that equilibrium of distribution of the tracer (Cl^{36}) has been reached, with the material being traced (Cl^{35}). Since it is impossible to determine when equilibrium of concentration has been obtained for every part or compartment of the body where chloride may be

found, it is necessary to employ an indirect approach. When an animal is in equilibrium and in a steady state with intake and output of the material being traced, and if at the same time the compartments into which the tracer is exchanging remain constant, then it is safe to assume after a single intravenous administration of the tracer that equilibrium of distribution has been reached when the rate of regression of serum concentration of the tracer is constant. This was determined in the present studies by calculating the slope of the curves of serum concentration (b values, described later). With the patient on a fixed regimen, equilibrium of distribution may be assumed, by this method, to exist when the slope is constant.[9]

From Fig. 2 it can be seen that the "b values" did not become constant until at least forty-eight hours after injection. Since serum samples were drawn only every twenty-four hours, it is not possible to determine the precise time at which equilibrium of distribution occurred but only to localize such time between successive twenty-four hour intervals. A relative constancy of slope was reached in sixty to seventy-two hours for the nonedematous subjects and in eighty-four to ninety-six hours for subjects with edema.

Comment.—Although an estimate of the time at which the b value becomes constant was obtained from Fig. 2, this does not necessarily indicate that complete equilibrium of distribution had been reached. In fact, it is conceivable that in many, if not all, instances true equilibrium of distribution may never be reached. However, for most practical purposes, in electrolyte studies at least, the time obtained by this method is sufficiently accurate.

In order to minimize errors in all calculations based upon need for attainment of equilibrium of distribution, we have assumed that equilibrium was not obtained until seventy-two hours had elapsed from the time of injection of the tracer. In the case of these Cl^{36} studies, although the data indicate that equilibrium of distribution was most likely completed by seventy-two hours, small subtle changes of distribution may still have been in progress beyond that time. However, the nature of the applications and interpretations of these data, such as biologic decay periods, are such that these slight variations would not alter the results and conclusions. These data indicate that calculations based on an assumption that equilibrium of distribution had occurred within twenty-four hours would be subject to serious error. Likewise, the effect of changing compartments would result in considerable inaccuracy.

BIOLOGIC DECAY RATES

$C_{\frac{1}{2}}$, $U_{\frac{1}{2}}$, and $E_{\frac{1}{2}}$ values were obtained for the six subjects. These parameters, which have been described previously,[1, 5] have the following connotations:

$C_{\frac{1}{2}}$ represents the length of time required for the concentration of the tracer material in the body fluid or substance or specific compartment to reach one-half the concentration existing at any time after equilibrium of distribution of the tracer has been reached. In this presentation $C_{\frac{1}{2}}$ refers to the serum compartment.

$U_{\frac{1}{2}}$ represents the length of time required for one-half the injected tracer material to be eliminated in the urine.

TABLE III. SUMMARY OF BIOLOGIC DECAY RATES FOR SIX HUMAN SUBJECTS STUDIED

SUBJECT NO.	PARAMETER	MEAN, ENTIRE STUDY	HIGH Cl INTAKE	LOW Cl INTAKE
A. Control				
501		Intake 73.12 meq./day	173 meq./day	31.4 meq./day
	$C_{½}$	15.0	6.9	25.1
	$U_{½}$	17.0	8.3	24.2
	$E_{½}$	14.9	8.0	23.4
504		Intake 85.2 meq./day	176 meq./day	28 meq./day
	$C_{½}$	8.04	4.76	33.24
	$U_{½}$	10.84	5.28	32.24
	$E_{½}$	7.02	3.26	30.51
B. Mild Congestive Heart Failure				
502		Intake 62.96 meq./day	153 meq./day	27 meq./day
	$C_{½}$	17.0	36.9	45.6
	$U_{½}$	50.6	41.2	44.4
	$E_{½}$	41.8	20.1	38.3
505		Intake 87.5 meq./day	175.7 meq./day	26.8 meq./day
	$C_{½}$	20.1	7.5	43.3
	$U_{½}$	16.9	9.0	92.4
	$E_{½}$	11.8	7.0	73.0
C. Severe Congestive Heart Failure				
503		Intake 62.11 meq./day	158 meq./day	31.2 meq./day
	$C_{½}$	71.5	22.1	68.0
	$U_{½}$	78.6	10.4	192.5
	$E_{½}$	44.1	7.0	141.5
506		Intake 79.5 meq./day		27.2 meq./day
	$C_{½}$	19.1		53.7
	$U_{½}$	22.4		315.1
	$E_{½}$	17.9		161.2

$E_{½}$ represents the length of time required for excretion of one-half the injected tracer by all measured routes, i.e., urine, feces, saliva, tears, and so forth.

These values are summarized by Table III and Figs. 3 through 11.

The average values for these three parameters were obtained by calculating mathematically the average of the daily slopes (the value of b) of the respective regression curves of serum concentration (C), urinary excretion (U), and total recorded excretion (E). The expression of the slopes was obtained from the following equation:

$$b_{1,2} = \frac{\log \frac{X_1}{X_2}}{t_2 - t_1}, \tag{1}$$

where X_1 is the value obtained by measurement of the various samples on one day (t_1) and X_2 on a later day (t_2). Since daily values for the slopes were calculated, $t_2 - t_1$ is unity, and the expression of the slope becomes

$$b = \log \frac{X_1}{X_2}. \tag{2}$$

In conformance with the practice of expressing biologic decay rates in terms of "half-life," the respective average b values were converted to mean $C_{½}$, $U_{½}$, and $E_{½}$ values. From the general equation

$$X_2 = X_1 e^{-bt} \tag{3}$$

it is obvious that when C, U or E becomes one-half of that obtained at the time of equilibrium of distribution, t_2-t_1 will equal $C_{½}$, $U_{½}$, or $E_{½}$, and $\frac{X_1}{X_2}$ will be 2. Therefore,

$$b = \frac{\log 2}{X_{½}} \text{ or } X_{½} = \frac{\log 2}{b} = \frac{0.693}{b}. \quad (4)$$

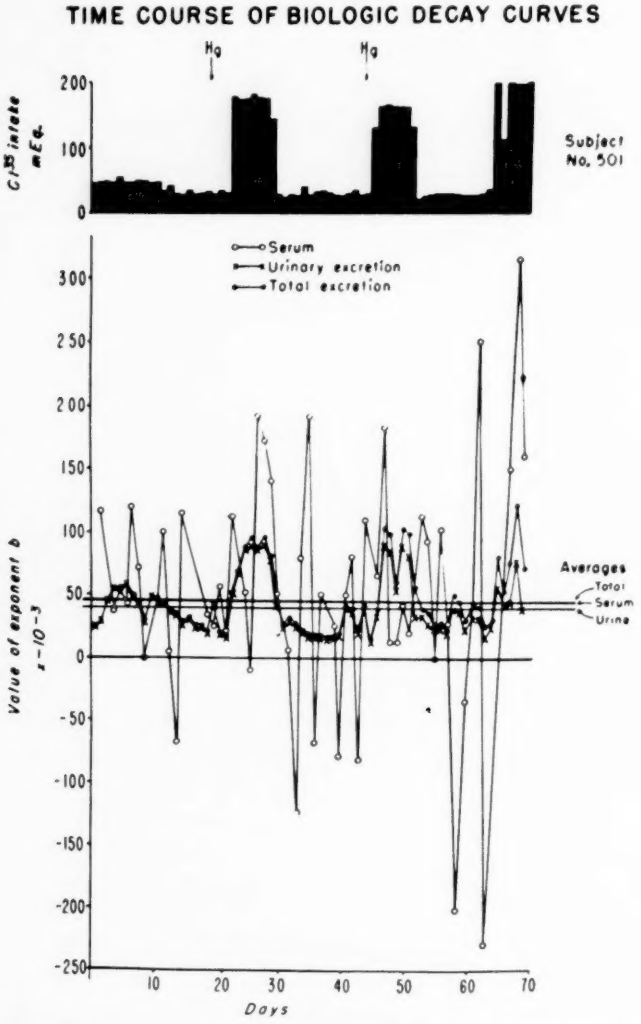

Fig. 3.—Daily variations in the slopes of the time curves of serum concentrations, urinary excretion, and total excretion of Cl^{36} for control Subject No. 501. Values above the abscissa are negative and those below, positive. Variations in the b values are related to the rate of Cl^{35} intake recorded at the top of the figure.

The biologic decay rates are extremely variable, reflecting many complex physiologic processes, including intake of the nontracer, drugs, and circulatory state. These values may be calculated for any period of time not only to define basal physiologic processes but also to evaluate the influences of modifying factors.

Figs. 3, 4, and 5 reveal wide daily variations in the respective biologic decay rates observed. The larger the magnitude of the negative b value, the more rapid is the rate of regression of the respective curves. A positive b value, which can occur only in the curve of serum concentration, results in an accession in the curve under consideration. Daily variations in the b values for one

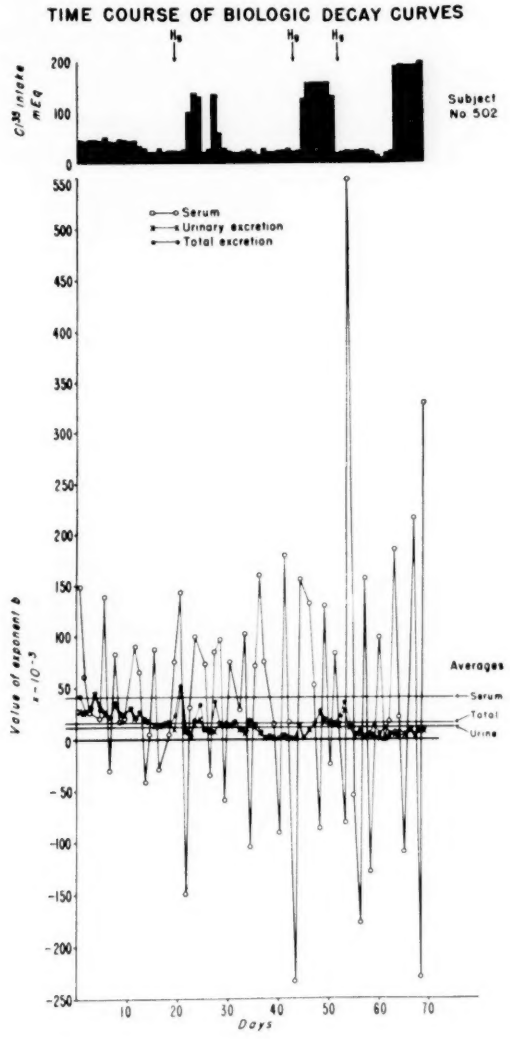

Fig. 4.—Data for Subject No. 502 with moderate chronic congestive heart failure. Method of presentation same as for Fig. 3.

of the control subjects are exemplified in Fig. 3, for a subject with mild to moderately severe chronic congestive heart failure in Fig. 4, and for a subject with severe congestive heart failure in Fig. 5. It can be seen from these illustrations that the b values of serum concentration, "C_b values," for subjects with congestive heart failure tended to vary more in magnitude than did those for the control subjects. This difference is particularly well emphasized by

comparison of the control subject and the one with severe heart failure (Figs. 3 and 5). The b values tended to be low in negative magnitude in the subjects with heart failure, due to depression in rate of turnover of the tracer and nontracer substances. However, when therapy resulted in diuresis and elimina-

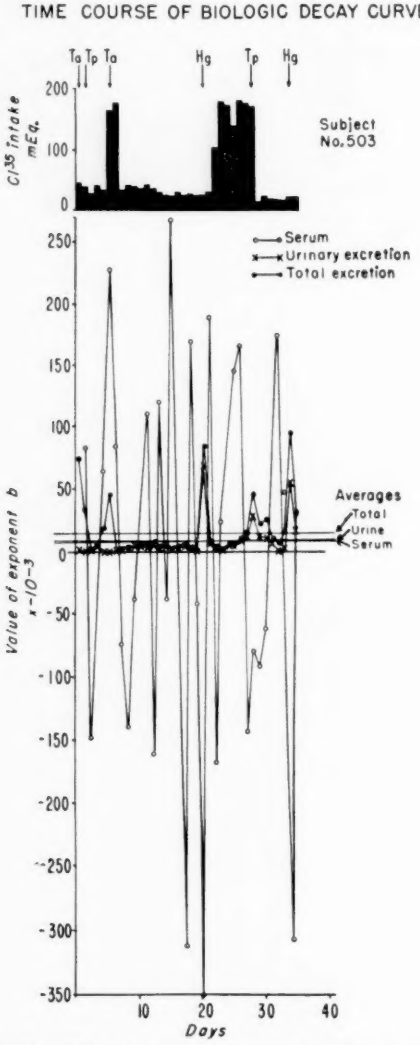

Fig. 5.—Data for Subject No. 503 with severe chronic congestive heart failure. Method of presentation same as for Figs. 3 and 4.

tion of edema fluid, the b values increased in negative magnitude, even exceeding the values for the control subjects. One subject with moderately severe chronic congestive failure whose degree of compensation improved during the period of observation had a mean b value which was essentially the same as that of the control subject on similar Cl^{35} intake. Figs. 3, 4, and 5 show that an increase in intake of Cl^{35} resulted in an increase in excretory rate and in magnitude of the negative value of b, except in the subjects with congestive

heart failure during periods of progressive decompensation. With compensation they, likewise, showed an increase in the b values or rate of turnover of chloride. In response to changes in chloride intake the b values varied in a manner similar to those of the control subjects.

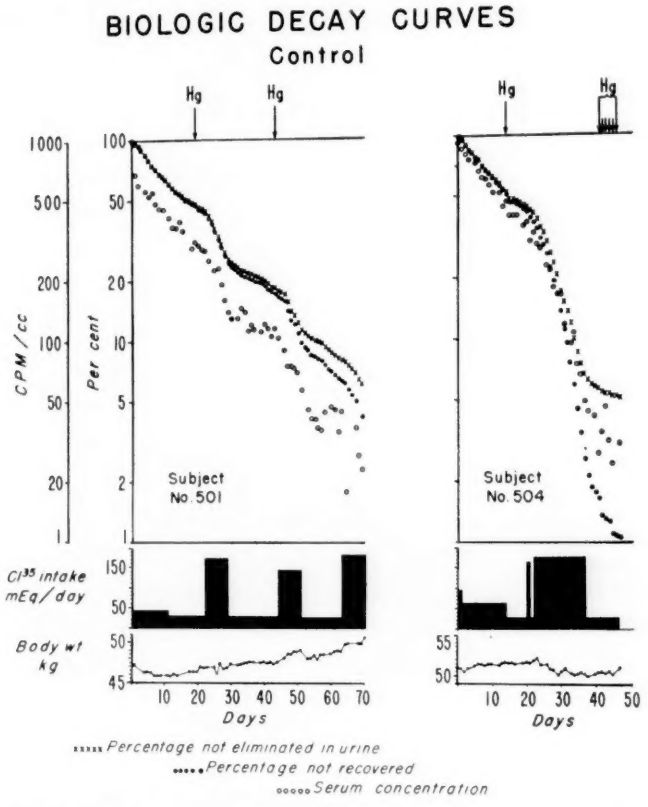

Fig. 6.—Time course of serum concentration, urinary excretion, and total excretion of Cl^{36} in two control subjects. The time course of urinary and total excretion of Cl^{36} are expressed as percentage of the administered dose of the tracer and not eliminated. The relationship of the curves to dietary intake of Cl^{35}, body weight, and mecurial diuretic are indicated by the illustration.

In general, the b values for blood concentration, urinary and total excretion varied concordantly in the control subjects, whereas numerous discordant variations in these values are noted for the subjects with heart failure. In the controls and in two of the four subjects with congestive failure the variations were more concordant during periods of high chloride intake than during periods of low intake. The peak values of U_b and E_b after mercurial diuresis were generally accompanied by a concordant variation in values of C_b in all subjects. For example, with establishment of a better state of compensation in Subject No. 505, the relation of the values of C_b, U_b, and E_b approached that of the control subjects.

The effect of various drugs is shown in Figs. 3, 4, and 5. Mercurial diuresis produced the largest negative values of U_b and E_b. Changes produced

by chloride intake are also apparent. The influence of drugs and other factors upon the biologic decay rates will be discussed in detail elsewhere.

The biologic decay rates are also illustrated by the curves of concentration-time course of the blood serum and by the curves of urinary and total recorded elimination (Figs. 6, 7, and 8 and Table III). The concentration-time course of Cl^{36} in the serum for the two control subjects reveals a more rapid rate of regression than for the subjects with congestive heart failure on a similar diet.

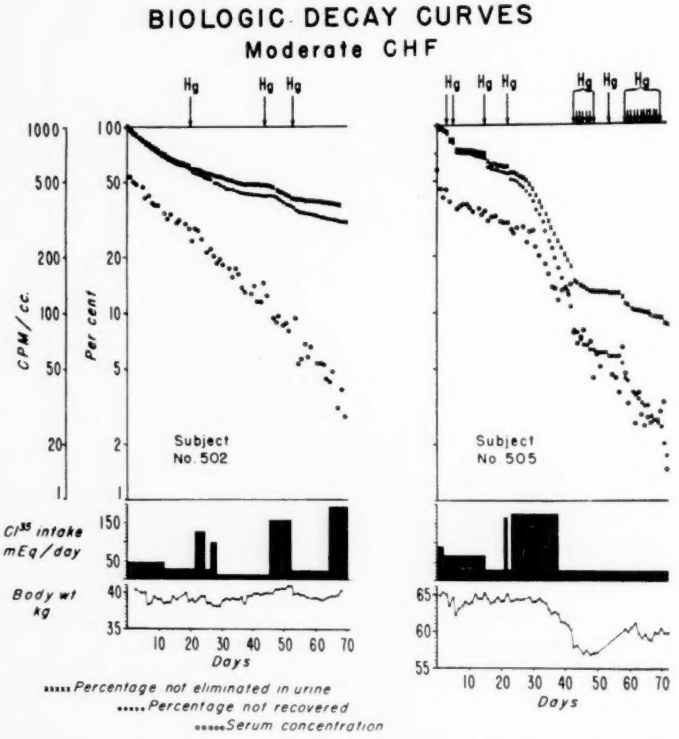

Fig. 7.—Time course of serum concentration, urinary excretion, and total excretion of Cl^{36} in two subjects with moderate chronic congestive heart failure. See text and legend of Fig. 6 for details.

The same was true for the two sets of curves of percentage tracer remaining in the body. While the control subjects were on a given regimen, there was little day-to-day variation in rates of regression of the three curves.

There were greater daily variations in the concentration-time course of Cl^{36} in the serum for the subjects with congestive heart failure than for the control subjects. These variations were especially wide in Subject No. 503, who had severe heart failure and died during the study. The rates of regression in serum concentration of Cl^{36} and the rates of regression of the two curves of excretion were definitely less rapid than those of the control subjects except when diuresis developed whether or not it occurred spontaneously or following therapeutic procedures (Figs. 6, 7, and 8). During periods of excessive diuresis these three curves regressed even more rapidly than did those of the control subjects.

Details of variations in rates of regression of these curves with respect to dietary intake of Cl^{35} are shown in Figs. 6, 7, and 8, but these relationships are better illustrated by Figs. 9, 10, and 11. Increased intake of Cl^{35} in the control subjects was accompanied by greater output and, therefore, more rapid rate of turnover of Cl^{36}, which is reflected in the increase in magnitude of -b. When

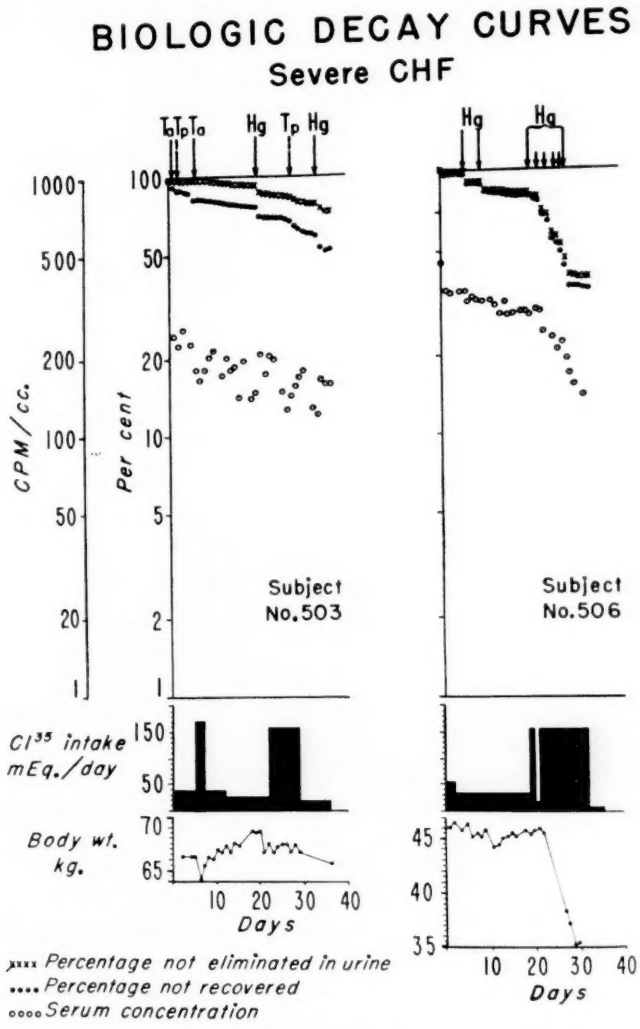

Fig. 8.—Time course of serum concentration, urinary excretion, and total excretion for two subjects with severe chronic congestive heart failure. See text and Fig. 6 for details.

the intake of Cl^{35} was reduced in the control subjects, the rate of turnover of Cl^{35} and Cl^{36} also decreased, as indicated by the decline in magnitude of -b (Fig. 9). Although the patients with congestive heart failure did not behave so predictably, they tended in general to behave like the normal subjects, that is, when the intake of Cl^{35} was increased, the magnitude of -b tended to become augmented (Figs. 10 and 11). Their response was influenced

by the state of the congestive heart failure, independently of the diet. The more the patient was compensated, the more nearly the response approached that of the control subjects in both direction and magnitude. When failure was extreme or progressing in severity, an increase in intake of Cl^{35} had little or no influence on the rate of turnover of Cl^{35} or Cl^{36} (Figs. 10 and 11). Furthermore, when diuresis developed, the rate of excretion of Cl^{35} and Cl^{36} increased, as did the magnitude of the –b values, even when the intake of Cl^{35} remained constant (Subject No. 505, Fig. 4).

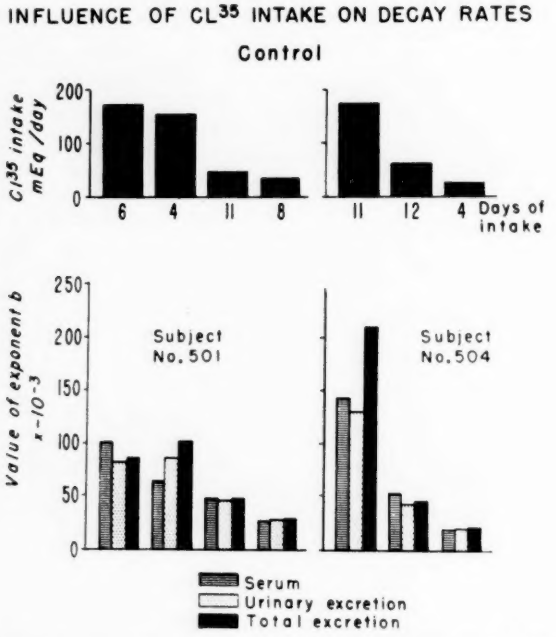

Fig. 9.—Illustration showing the relation of dietary intake of Cl^{35} to rate of decline in serum concentration, and rates of urinary and total excretion of Cl^{36} for the two control subjects. The greater the value of negative b the greater was the decline in serum concentration of Cl^{36} or the greater was the rate of excretion of the tracer.

The general rates of biologic decay are presented in Table III. Although this is the more conventional method of presenting the rates of biologic decay, the data in Table III are not nearly as detailed as the previously presented b values or regression curves themselves.

(a) *Normal Subjects.*—During the entire period of study the average $C_{\frac{1}{2}}$ values for the two control subjects were fifteen and eight days, respectively, the $U_{\frac{1}{2}}$ values were seventeen and ten and eight-tenths days, and the $E_{\frac{1}{2}}$ values fourteen and nine-tenths and seven days, respectively. When these subjects were placed on a high intake of chloride (173 and 176 meq. per day, respectively), these parameters changed considerably, indicating more rapid rates of biologic decay. $C_{\frac{1}{2}}$ for the two subjects changed to six and nine-tenths and four and eight-tenths days, $U_{\frac{1}{2}}$ to eight and three-tenths and five and three-tenths, and $E_{\frac{1}{2}}$ to eight and three and three-tenths days, respectively. When their intake of chloride was low (31 and 28 meq. per day, respectively), the biologic decay rates

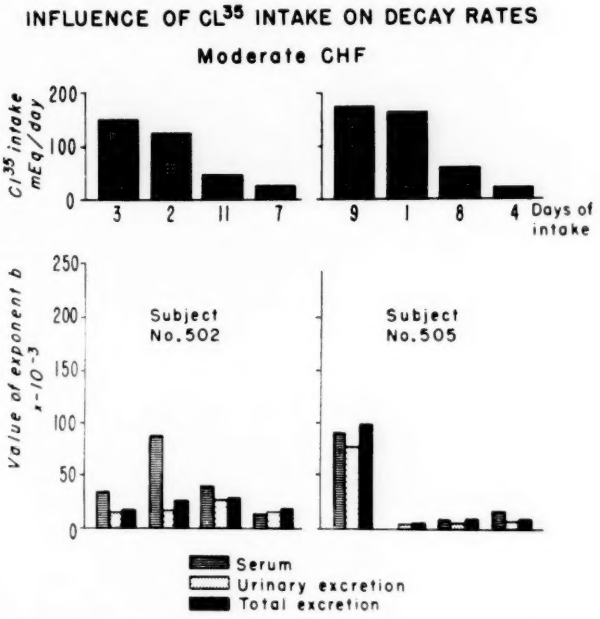

Fig. 10.—Illustration showing the relation of dietary intake of Cl^{35} to rate of decline in serum concentration and rates of urinary and total excretion of Cl^{36} for the two subjects with moderately severe chronic congestive heart failure. The greater the value of negative b, the greater was the decline in serum concentration of Cl^{36} or the greater was the rate of excretion of the tracer.

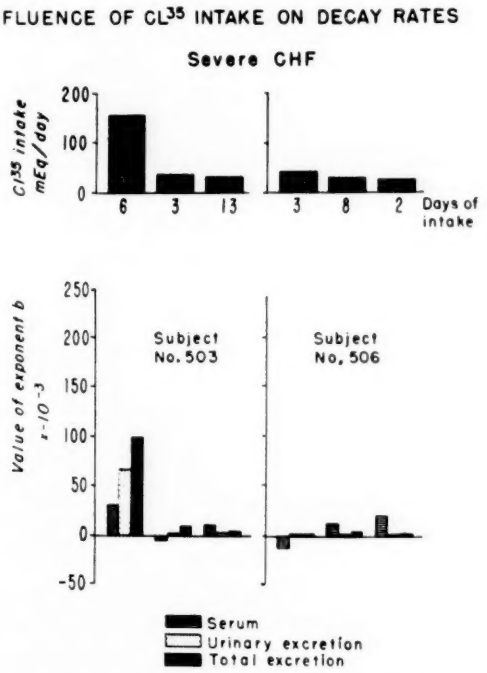

Fig. 11.—Illustration of the relation of the dietary intake of Cl^{35} to the rate of decline in serum concentration and rates of urinary and total excretion of Cl^{36} in two subjects with severe chronic congestive heart failure. The greater the value of negative b, the greater was the decline in serum concentration and the greater was the rate of excretion of the tracer.

TABLE IV. METHOD OF PREDICTION OF $C_{1/2}$ VALUES FROM OBSERVED DATA

A. Observed Data

CODE LETTER	ANIMAL	WEIGHT (KG.)	$C_{1/2}$ (DAYS)	Cl^{35} INTAKE I (GM.)	Cl^{35} MASS Mo (GM.)	PER CENT INTAKE OF Cl^{35} MASS I/Mo × 100	NaCl (GM.)
a	Human Subject No. 501	47.268	15	2.596	55.85	4.68	4.28
		70		3.84	82.05		6.34
b	Human Subject No. 504	50.904	8.04	3.02	37.58	8.04	4.98
		70		4.15	51.6		6.85
c	Dogs, Mean Values	4.767	2.51	1.96	6.52	30.06	3.23
		4.545		1.87	6.22		3.09
Ratio							
b:a			0.536	1.081	0.629	1.718	
c:a		0.065	0.167	0.487	0.075	6.423	
c:b		0.065	0.312	0.450	0.121	3.739	

B. Predicted Data

DATA USED FOR PREDICTION	METHOD OF PREDICTION	ACTUAL VALUES USED	PREDICTED $C_{1/2}$	OBSERVED $C_{1/2}$	ERROR IN PREDICTION
a predicted from b values	(Observed $C_{1/2}$ for b) × (per cent intake of Cl^{35} mass for ratio b:a)	8.04 × 1.718	13.81	15.0	−7.9%
a predicted from c values	(Observed $C_{1/2}$ for c) × (per cent Cl^{35} intake of Cl^{35} mass for ratio c:a)	2.51 × 6.423	16.1	15.0	+7.3%
b predicted from a values	Observed $C_{1/2}$ for a) ÷ (per cent Cl^{35} intake of Cl^{35} mass for ratio b:a)	15 ÷ 1.71	8.71	8.04	+8.3%
b predicted from c values	(Observed $C_{1/2}$ for c) × (per cent Cl^{35} intake of Cl^{35} mass for ratio c:b)	2.51 × 3.739	9.38	8.04	+16.4%
c predicted from a values	Observed $C_{1/2}$ for a) ÷ (per cent Cl^{35} intake of Cl^{35} mass for ratio c:a)	15 ÷ 6.423	2.33	2.51	−7.2%
c predicted from b values	Observed $C_{1/2}$ for b) ÷ (per cent Cl^{35} intake of Cl^{35} mass for ratio c:b)	8.04 ÷ 3.739	2.15	2.51	−14.3%

decreased strikingly, the $C_{1/2}$ values changing to twenty-five and one-tenth and thirty-three and two-tenths days; the $U_{1/2}$ values to twenty-four and two-tenths and thirty-two and two-tenths days, and the $E_{1/2}$ values to twenty-three and four-tenths and thirty and five-tenths days, respectively.

(b) *Patients with Moderately Severe Congestive Heart Failure.*—The average biologic decay rates for the entire period of study were: $C_{1/2}$, seventeen and twenty and two-tenths days; $U_{1/2}$, fifty and six-tenths and sixteen and nine-tenths days, $E_{1/2}$, forty-one and eight-tenths and eleven and eight-tenths days. When these patients' intakes of chloride were high (196 and 175 meq. per day, respectively), these values changed as follows: $C_{1/2}$, nine and two-tenths and seven and five-tenths days; $U_{1/2}$, 105 and nine days; $E_{1/2}$, seventy-four and five-tenths and seven days, respectively. Thus in one subject the rates increased and in the other there was a significant decrease.

When these subjects were on a low intake of chloride (27 meq. per day for both) the biologic decay rates were: $C_{\frac{1}{2}}$, forty-five and six-tenths and forty-three and three-tenths days; $U_{\frac{1}{2}}$, forty-four and four-tenths and ninety-two and four-tenths days; $E_{\frac{1}{2}}$, thirty-eight and three-tenths and seventy-three days, respectively. The rates of one subject decreased during low Cl^{35} intake, whereas those of the other patient, which declined sharply on high intake, decidedly increased when intake of chloride was low.

(c) *Patients with Severe Chronic Congestive Heart Failure.*—The average biologic decay rates for the entire period of observation on two subjects in this category were: $C_{\frac{1}{2}}$, seventy-one and five-tenths and nineteen and one-tenth days; $U_{\frac{1}{2}}$, seventy-eight and six-tenths and twenty-two and four-tenths days; and $E_{\frac{1}{2}}$, forty-four and one-tenth and seventeen and nine-tenths days, respectively. During periods of relatively high chloride intake (158 meq. per day), the rates were: $C_{\frac{1}{2}}$, twenty-two and one-tenth and eighteen and seven-tenths days; $U_{\frac{1}{2}}$, ten and four-tenths and 157.5 days; and $E_{\frac{1}{2}}$, seven and 141.5 days, respectively. On low chloride intake (31 and 27 meq. per day), these parameters indicated a much slower rate of exchange: $C_{\frac{1}{2}}$, sixty-eight and fifty-three and seven-tenths days; $U_{\frac{1}{2}}$, 192.5 and 315.1 days; and $E_{\frac{1}{2}}$, 141.5 and 161.2 days, respectively.

COMMENT

The data obtained in this study indicate a conspicuous difference between the renewal or turnover rates of control subjects and patients with congestive heart failure. It is apparent from Table III and Figs. 3 through 11 that subjects with heart failure excrete chloride more slowly than normal subjects on a comparable diet without the influence of diuretics. It is also apparent that the biologic half-life is influenced by the presence as well as the degree of the abnormality, since the subjects with severe congestive failure had slower turnover rates than did those with mild failure. The magnitude of these differences was roughly comparable to those obtained with the use of Na^{22} in similarly designed experiments.[1] In the present experiments subjects without congestive heart failure were selected as control subjects. That the excretion and renewal rates are the same in these and perfectly healthy individuals is doubtful. Some information would suggest that certain forms of tuberculosis are associated with disturbances in chloride metabolism.[10] Control Subject No. 504 had clinically inactive tuberculous lymphadenitis. Regardless of this, there are apparent differences between rates of excretion and turnover for the control subjects and for subjects with either mild or severe congestive heart failure.

Restriction of chloride intake in control subjects will alter their curves depicting excretion and turnover to resemble those of patients with congestive heart failure; yet with similar dietary intake the curves will differ. This same type of response was observed with Na^{22}. The terms "similar" or "comparable" diets usually refer to absolute quantitative intake of the nontracer in milliequivalents or in grams without relation of this intake to the total amount of this element in the body. If, however, the diet of the control subject contained the same percentage per day of the total body content of Cl^{35} as that of the

patient with heart failure, then the renewal rates would be identical, provided there were no change in body content of Cl^{35}. Since congestive heart failure is associated with an accumulation of extracellular fluid and since the concentration of NaCl in extracellular fluid is high, it is conceivable that if both subjects have the same weight, the patient with failure would require a greater daily intake of chloride to obtain the same percentage of body chloride daily as the nonedematous control subject. The difference in quantity required would depend on the amount of extracellular fluid present. This would also be true for the other principal constituents of extracellular fluid, such as Na^{23}. Observations with Na^{22} support this point.[1] When the element being traced is primarily intracellular, differences in intake would not have to be so great to be "comparable," since the total body content of intracellular constituents is not increased by an accumulation of extracellular fluid during episodes of congestive heart failure.

The relation of intake of nontracer Cl^{35} to percentage excretion of tracer Cl^{36} depends upon several conditions. One of the principal factors necessary for an absolute relationship to exist is that all of the element being traced must be in compartments which have free communication with all other compartments of Cl^{35} in the body. Furthermore, there must be no absolute gain of the nontracer by either increased weight or local shifts within the body. Thirdly, the intake of the nontracer must not exceed the excretory capacity of the subject for any period of time, a factor especially important for the patient with congestive heart failure. Since patients with congestive heart failure are known to have limited excretory capacity for electrolytes, it is easy to overload them and thereby produce an absolute gain of the nontracer substance in the body. If all compartments are in free exchangeable communication, no gain in total body content of nontracer and no selective local shifts within the body occur, so that when the intake is increased by a factor of 5, one would expect a reduction in the values of $C_{\frac{1}{2}}$ by a factor of 5. This would hold even for the subjects with congestive failure, if the foregoing conditions are maintained. This relationship, however, would not hold in the presence of an absolute gain or loss of the nontracer. Inspection of Table III reveals that increasing the intake of Cl^{35} by a factor of 5.5 in control Subject No. 501 reduced $C_{\frac{1}{2}}$ by a factor of 3.6, and a 6.3-fold increase in diet for control Subject No. 504 reduced $C_{\frac{1}{2}}$ by a factor of 6.9. In one subject with mild failure, a 6.5-fold increase in intake lowered $C_{\frac{1}{2}}$ by a factor of 5.7. In one subject with severe failure, a 5-fold increase in Cl^{35} intake produced a reduction in $C_{\frac{1}{2}}$ by a factor of 3.08. These relationships were derived from the periods of high and low dietary intake of chloride, reflecting essentially the effect of intake alone on the biologic half-life. The mean values for the entire period of study reflect the influence not only of varying intake but also of therapeutic procedures such as paracentesis, mercurial diuretics and other drugs and are therefore not suitable for comparison with values for isolated periods of known intake. However, from these relationships derived from observations obtained during periods of high and low dietary intake of chloride, it is apparent that although the controls did not entirely satisfy the conditions, they did so more nearly than the patients with severe

failure. Such lack of exact relationships might be due to several factors. Evidence has accumulated that a subcompartment may cease to exist as a freely exchangeable unit among the other Cl^{35} compartments in the body. This concept is supported by the sharp rises in serum concentration of Cl^{36} after a sudden increase in intake of Cl^{35}. The latter may have "reopened" a compartment which became equilibrated with a high concentration of Cl^{36} and later effectively "closed off." Reopening of this compartment with a subsequent reentry of this Cl^{36} into the serum would result in a rise in serum concentration. Such a set of circumstances may be noted in Figs. 6, 7, and 8, which are not explained by loss of water out of proportion to the electrolyte, inasmuch as serum Cl^{35} did not increase. Further evidence for the "blinking" of subcompartments from the behavior of the $C_{\frac{1}{2}}$ values with variations in dietary intake of Cl^{35} will be discussed in detail elsewhere.[11]

That the normal relationship of intra- and extracellular chloride may be disturbed in congestive heart failure is indicated by inspection of Figs. 6, 7, and 8, which reveal sharp declines in serum concentration unaccompanied by any gain in weight or by increased excretion of Cl^{36}. Such a disturbance, i.e., a fall in serum concentration of Cl^{36} not attributable to excretion and not explainable on the basis of a simple dilution through increase in extracellular fluid, must be due to a shift of chloride to the intracellular compartment or adsorbed onto cells or complex molecules as proteins or lipoproteins. Subject No. 504 presents findings in support of such concepts. When she was given a 3 per cent solution of NaCl intravenously, there was a fall in serum concentration of Cl^{36} from 363 to 298 CPM per milliliter (Fig. 6). The serum Cl^{35} varied from 97 to 99 meq. per liter. Body weight was unchanged and urinary volume declined. There was an increase in urinary excretion of Cl^{35} from 31½ to 50 meq. per day. Urinary excretion of Cl^{36} increased from 100,000 to 162,000 counts per minute in twenty-four hours, but this was not sufficient to explain the fall in serum concentration. These data were interpreted to indicate a shift of extracellular chloride to the intracellular compartments or reopening of a compartment into which Cl^{36} had not previously diffused. Instances of this same phenomenon may be found in the subjects with congestive heart failure. Evidence has been obtained that Cl^{35} may shift into certain cells of the body under influences of adrenal insufficiency. Stern and his associates found an increase of 20 per cent in Cl^{35} in muscle, despite a decrease in total extracellular Cl^{35}. This phenomenon of shifting from extra- to intracellular compartments will be considered in detail elsewhere.[11]

It was noted that patients with congestive failure tended to show considerable instability of serum concentration of Cl^{36} (Figs. 6, 7, and 8). These fluctuations were out of the range of technical errors. Such observations indicate that the patients with congestive failure did not possess the fine regulatory control of serum concentration of chloride as did normal people. Figs. 3, 4, and 5 demonstrate this extreme variability for the subjects with congestive heart failure. The b values for urinary and total recorded excretion show some

variation, but the C_b values are impressive by the extreme day-to-day alterations. The precise location of the fault in such cases is unknown. It is probable that other substances as well vary as much or even more. Such variations in the proper substances are probably related to some of the symptoms and signs encountered in congestive heart failure, as myasthenia and neurasthenia.

Although this presentation is concerned chiefly with the influence of chloride intake on the biologic decay periods, the effect of certain drugs is also apparent in Figs. 6, 7, and 8. Rates of excretion of Cl^{36} may rise considerably with mercurial diuresis, as occurred in Subject No. 506. Equally striking is the effect of improvement in state of compensation, as exhibited by Subject No. 505 (Fig. 7). With improvement and coincident increased intake of chloride, there was a rapid rate of chloride turnover. The type of excretory curve seen in this subject might be explained by a changing intake of Cl^{35}, such as when intake is progressively increasing. However, in this case the intake was constant during this interval. Two other mechanisms might be operative to produce such a phenomenon. If the subject were previously depleted of chloride, the initial period of increased intake would in part be filling "cavities" or partially depleted chloride spaces and could possibly act as though the spaces were becoming progressively more filled. In addition, a constant intake could effectively behave as a progressively increasing magnitude of intake if the total body weight and, therefore, the total body chloride space were decreasing during the interval under consideration. In this way the constant intake would be a progressively greater percentage of the total body content of the element. There would be a progressive increase in "effective intake." In Subject No. 505 there was considerable weight loss during this interval of time, so the latter mechanism might have been responsible for an excretion curve which shows a progressively increasing rate of excretion. Control Subject No. 504 had a low chloride content of serum and presented a somewhat similar type of curve. In this instance the curve may have resulted from filling "cavities" with the first increments of the high intake, thus acting as a progressive increase of effective intake. The serum chloride rose to normal in this subject during the interval under consideration.

During periods of low intake a subject with congestive heart failure may have a biologic half-life which seems short in proportion to the intake. This is especially true for $U_{\frac{1}{2}}$ and $E_{\frac{1}{2}}$. With restriction of electrolyte intake well within the excretory capacity of the individual, there will be an excretion out of proportion to intake, loss of weight and absolute loss of chloride from the body. Under such circumstances, if chloride is lost in such a fashion to maintain isotonic concentration in the remaining extracellular fluid and the normal serum concentration of Cl^{35}, $C_{\frac{1}{2}}$ will not fall as rapidly as $U_{\frac{1}{2}}$ and $E_{\frac{1}{2}}$. Thus, with sufficient dietary restriction or with improvement of the state of compensation, the ensuing diuresis may result in apparent discrepancies between $C_{\frac{1}{2}}$, $U_{\frac{1}{2}}$, and $E_{\frac{1}{2}}$. If, however, intake exceeds the excretory capacity of the individual and there results an accumulation of extracellular fluid with an absolute gain of chloride by the body, then the half-life measurements may exhibit a discrepancy in the opposite direction, i.e., $C_{\frac{1}{2}}$ will be much shorter

than $U_{½}$ and $E_{½}$. This would be the result of simple dilution of the tracer. These and other aspects of tracer measurements have been considered in detail elsewhere.[12]

In some of the subjects there were instances in which $E_{½}$ was much shorter than $U_{½}$. The absolute value of $E_{½}$ can be no greater than $U_{½}$ and may be considerably shorter when there is significant loss of chloride by vomiting, diarrhea, or removal of pleural or peritoneal fluid (Figs. 3, 4, 5, 6, 7, and 8 and Table III). In Figs. 3, 4, and 5 certain discrepancies in magnitude of b values for U and E are apparent. When mercurial diuresis occurred, magnitude of E_b and U_b increased proportionately, but when ascitic fluid was removed the magnitude of E_b became much larger than that of U_b.

GENERAL DISCUSSION

With the use of a long-life isotope it has been possible to make certain observations on chloride metabolism which were not previously possible. Additional information was also obtained which could be determined with short-life isotopes (Cl^{38}) and even by chemical methods. Previous studies of rapid diffusion with Cl^{38} have been reported.[13, 14] Unfortunately, the usefulness of this inexpensive and readily available isotope is limited by its short half-life, which precludes observations on phenomena of chloride metabolism and turnover. During the course of the use of the long-life isotope it was possible to observe readily many aspects of chloride metabolism; results obtained in these experiments will be reported at a later date.

The time of occurrence of equilibrium of distribution of the injected isotope with the previously existing Cl^{35} in the body may be ascertained by use of the long-life isotope. Previously this had been estimated with the use of Cl^{38}, but the data presented in this report indicate that this estimation is inaccurate. Equilibrium of distribution of Cl^{36} is apparently not accomplished before at least forty-eight hours after injection[9]; therefore any estimation of chloride space prior to the first forty-eight hours following injection of the tracer is likely to be erroneous. Furthermore, the time at which such equilibrium of distribution occurs may depend upon the amount of extracellular fluid present. In determination of equilibrium of distribution, it is assumed that the injected tracer has free communication with all existing compartments in the body, but this may not be the case. Previous studies[15, 16] indicate that all the nontracer may not be "tagged." If the dietary intake of Cl^{35} has been low and some of the Cl^{35} compartments in the body have closed off, as the data presented suggest, then complete equilibrium of distribution would not occur in the expected time, and space measurements would be considerably inaccurate.

Observations in control subjects and in patients with various degrees of congestive heart failure revealed that the latter have slower turnover rates. The more severe the heart failure, the slower are the rates of turnover. The rates of turnover of Cl^{36} among normal subjects and patients with various degrees of heart failure were roughly comparable to those obtained in similar subjects with the use of Na^{22}. This would be expected, since the compartments of the two elements are similar in location and extent.

The rate of turnover of chloride in man and dog, at least, and most probably in any metabolic compartment,[12] must be determined by the rate of intake of the chloride and the mass of chloride in the compartment. Careful study of Table IV will support this point. Table IV is divided into two parts: (A) data obtained by direct observation and (B) predicted data. The data obtained by experiment include weight, $C_{\frac{1}{2}}$ values, intake of Cl^{35}, mass of chloride in the body determined by dilution of injected Cl^{36}, percentage that Cl^{35} intake represents of total body mass of Cl^{35}, and Cl^{35} intake as NaCl for two human control subjects and the average values for five normal dogs. The ratios at the bottom of the table are indicated by code letters and are for the values shown in the respective columns above. Table IV, B, concerned with prediction of $C_{\frac{1}{2}}$ values, shows how, from observation of turnover rate of Cl^{36} of any one subject and with a knowledge of intake and body mass of Cl^{35} of this subject and of another subject, it is possible to predict within about 10 per cent accuracy the rate of turnover of the other subject. This can be estimated for man from turnover data on dogs or for dogs from turnover data on man. It is also interesting to note that the same can be done for Cl^{35} from turnover data obtained with Na^{22} [1] and vice versa.[5]

Such satisfactory quantitative correlation of data summarized in Table IV indicates the adequacy and accuracy of the experimental data obtained, both for the rates of turnover and for the mass of Cl^{35} calculated from the dilution of the injected Cl^{36}. The methods for measuring the rates of turnover of Cl^{36} as well as other electrolytes have been described, and those for measuring the body mass of Cl^{35} will be presented and discussed in detail elsewhere.[11]

These experiments show that chloride is not excreted as rapidly by patients whose chronic congestive heart failure is progressively becoming worse as by normal subjects. The mechanism for this difference is not known. However, once compensation begins, the turnover increases directly with the degree of compensation. The use of therapeutic agents, as for example mercurial diuretics, may produce diuresis and consequently alter the biologic decay rates of chloride. Other modifying factors, such as bed rest, digitalis, and dietary intake of chloride, are so numerous and the moment-to-moment variations are so large in response even to the same factor in a single subject or in different subjects that careful control of these experiments becomes essential. Even in the most carefully controlled studies, the mechanism of responses is difficult to interpret. Disease states complicate the problem even more because spontaneous variation in the state of the disease occurs independently of variables intentionally introduced. Furthermore, the methods employed in these experiments are relatively insensitive to the many known and unknown subtle physiologic processes which have indirect governing influences upon the results, thus rendering detailed interpretations of the data more difficult or even impossible. Regardless of these difficulties, however, gross changes are sufficiently well recorded to yield important information, provided no attempt is made to explain the mechanisms in detail.

The significance of the biologic decay rates from the radiobiologic and military points of view is self-evident, and therefore only certain aspects will

be indicated briefly. To determine the dosage of radiation from radioactive chloride over a given period, one must know the duration of exposure. The physical characteristics of the radiation are already known and now, with some knowledge of the duration with which the isotope remains in the body under various dietary and clinical conditions, it is possible to calculate more accurately the total radiation dosage. Furthermore, from detailed analysis of the illustrations and tables one may obtain additional information applicable to his own particular needs. It is also possible not only to evaluate the influence of congestive heart failure and generalized edema on the safe amount of exposure to the radiation but also to predict the response to mercurial diuretics and high dietary intake of chloride and their influence on reducing exposure to radiation by increasing the rate of excretion of the isotope. The importance of these data and methods of influencing biologic decay rates in the event of atomic warfare is evident.

SUMMARY

Various biologic decay rates, $C_{\frac{1}{2}}$, $U_{\frac{1}{2}}$, and $E_{\frac{1}{2}}$, were determined by prolonged observation of six human female subjects with the use of the long-life radioactive isotope of chloride, Cl^{36} ($t_{\frac{1}{2}}$ 4.4×10^5 yrs.). Two of the subjects were controls, two had moderately severe chronic congestive heart failure, and two had severe chronic congestive heart failure.

In one control subject the rate of transfer of Cl^{36} from the blood serum was found to be defined by the equation,

$$CN_t = 10600e^{-2.496t} + 935e^{-0.3097t} + 860e^{-0.0013t}$$

Equilibrium of distribution of the Cl^{36} was estimated to have been reached sometime after the first forty-eight hours and most probably by seventy-two hours following injection for the nonedematous subjects. For safer assumption, three days would be the more acceptable time, and five days may be required for detailed and accurate investigations based upon establishment of equilibrium of distribution, especially for subjects with edema.

The $C_{\frac{1}{2}}$, $U_{\frac{1}{2}}$, and $E_{\frac{1}{2}}$ values of rates of biologic decay were studied in detail on a daily basis as well as over longer periods of time. The daily variations were considerable, especially in one or two patients with chronic congestive heart failure and probably reflected the physiologic variations of the milieu interieur. The role which this plays in physiologic expressions of congestive heart failure remains obscure. About eight to fifteen days were required in the control subjects for turnover of one-half the chloride in the body, whereas in one patient with chronic congestive heart failure seventeen days were required and in another seventy-two days during a period of average chloride intake. A moderately high intake increased the turnover rates considerably and a low intake reduced them strikingly. In the patients with failure the rate increased during compensation and fell during decompensation, the state of the failure and therapy influencing the rates remarkably.

From a knowledge of the body mass of chloride and intake of chloride in man and dogs it was possible to predict the turnover rates in normal dogs from the rates measured for human control subjects and vice versa. The significance and theoretic and practical implications of these studies are discussed.

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SERIAL OBSERVATIONS OF I^{131} LEVELS IN THE PLASMA AS AN AID TO THE DIAGNOSIS OF HYPOTHYROIDISM

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RADIOIODINE has been used widely for the detection of thyroid disease during the past few years. Its use in this respect may be divided into the various functions of the thyroid that may be measured with it.

Table I lists certain functions of the thyroid with tests that seem to measure them most accurately with radioiodine.

Table I

FUNCTION	TEST
1. Cellular hyperplasia (acinar cell height)	Uptake gradient[3] 24-hour uptake
2. Storage of newly formed hormone	24-hour uptake followed by thiocyanate or iodine[3]
3. Secretion of thyroid hormone	Rate of appearance and partition of radioactive iodine into a "protein-bound fraction" (in blood or plasma)[6]
4. Retention of iodine by the body	Urinary excretion studies with I^{131}[1, 4, 5]
5. Rate of utilization of thyroid hormone	"Disappearance" of protein-bound radioactive iodine into a "protein-
6. Rate of iodine absorption to, and loss from, the blood	Radioactive iodine blood levels[8, 9]

None of these tests represents a definitive test of the clinical syndromes associated with thyroid disease—they represent certain features of thyroid behavior that may be associated with the clinical condition.

The familiar uptake study is probably no better guide to clinical states than is the histologic examination of thyroid tissue.[3] Because of the difficulty which had been found in this clinic in separating certain borderline states of thyroid disease by simple uptake and excretion studies, the measurement of serial plasma levels of radioiodine was undertaken. It was hoped that such radioactive iodine tolerance curves might prove a useful diagnostic aid.

METHODS AND MATERIALS

The technique used in measurement of plasma samples has already been described in detail.[8] Briefly—following the postfasting ingestion of 100 μc of radioactive iodine, blood was drawn from thirty-six subjects at thirty minutes, and one, three, six, twelve, twenty-four, and forty-eight hours. In addition, one hypothyroid, five euthyroid, and four hyperthyroid cases had blood drawn at six, twelve, eighteen, and twenty-four minutes, and at seventy-two and ninety-six hours, and eight and sixteen days. Suitably prepared plasma samples were assayed for radioactivity. The thirty-six patients used were divided as follows: 13 hypothyroid (11 women, 2 men—ages 24 to 74 years); 11 hyperthyroid (7 women, 4 men—ages 33 to 66 years); 12 normal (3 women, 9 men—ages

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22 to 52 years); all patients were classified following clinical evaluation plus conventional studies such as: protein-bound iodine, basal metabolic rate, and blood cholesterol determinations. No patient had received treatment or drugs which might interfere with radioiodine studies.

The amount of radioactivity in duplicate 0.2 ml. specimens of plasma was recorded directly as counts per minute—no attempt was made to relate the amount appearing to the dose given. When serial counts were thus obtained from a patient they could be plotted in graphic style—it was with the shape of these curves that this study was concerned. The counts shown were all corrected for physical decay. Composite curves of the three groups of patients were calculated from the geometrical mean of plasma counts of each time interval plotted against time on a logarithmic basis. The "normality" of the data was checked by Probit analysis.* No effort was made to relate the curves to the "specific activity" of the radioiodine. Twenty-four-hour thyroid pickup studies were done by comparing the amount of radiation detected with a shielded Geiger tube, from the thyroid, with the radiation detected in a 50 ml. dummy flask containing the same amount of I^{131} originally ingested by the patient.

The urinary excretion studies were performed on 0.2 ml. aliquots obtained from the twenty-four- and forty-eight-hour urine specimens and compared with similarly prepared 0.2 ml. aliquots from a dummy specimen containing the same quantity of I^{131} as was ingested by the patient.

RESULTS

Fig. 1 shows three composite curves drawn from the geometric means of the three groups of patients—hypothyroid, hyperthyroid, and euthyroid. The data, prior to thirty minutes and after seventy-two hours on which these curves are based, are small; further exploration of these intervals is warranted. The maximum difference in configuration is noted between the myxedematous and the two nonmyxedematous groups. The peak of the myxedema curve is lower and occurs later than that of the nonmyxedematous groups. The complete listing of counts per minute at each time interval is in Tables II, III, and IV. The separation of the myxedematous and nonmyxedematous groups is best demonstrated at six, twelve, twenty-four, and forty-eight hours. The general

TABLE II. NORMAL SUBJECTS, SHOWING COUNTS PER MINUTE OBSERVED IN 0.2 C.C. PLASMA AT VARIOUS TIME INTERVALS AFTER INGESTION OF 100 MICROCURIES OF I^{131}

	TIME														
	MINUTES					HOURS								DAYS	
PATIENT	6	12	18	24	30	1	3	6	12	24	48	72	96	8	16
R. A.					860	480	363	194	90	31	0				
J. B.	86	408		543	518	398	231	129	62	28	0				
H. C.	64		256	509	671	676	443	236		45	20				
J. C.					731	822	636	248	128	70	24				
G. G.					635	446	313	170	62	10	0				
R. H.			198		398	350	218	120	71	19	7				
C. H.			596		600	530	363	272	91	25	4				
D. P.					460	372	291	172	118		69			72	
L. S.					704	638	499	247	128	121	63				
J. H.	43	120	322	613	739	542	344	168	85	21	5	7	10	6	32
B. C.	23	120	239	260	248	456	256	167	92	39	4	13	4	4	4
N. F.	125	287	334	339	381	484	245	208	87	28	6.5	10	16	6	7
Geometric mean of counts	58	203	303	431	560	501	333	189	89	32	13	10	9	6	10

*Acknowledgment is made of the help given in statistical analysis by Dr. Frederick T. Moore.

configuration of the curves is of more importance than the absolute counts at any one time. Fig. 1 also shows the composite curves of the normal subjects and of the hyperthyroid patients. These curves show only suggestive differences—an earlier and lower peak in hyperthyroid than in normal cases, and a more rapid drop in serum level in the hyperthyroid cases; the hyperthyroid curves show a definite rise after twenty-four to forty-eight hours. Nine of twelve euthyroid cases had their maximum peak of activity at thirty minutes, the remaining three at one hour. Of four cases of hyperthyroidism, the maximum level of serum activity was before thirty minutes. Three normal and three hyperthyroid patients were followed for seventy-two and ninety-six hours and eight days; the serum of the hyperthyroid patients showed a definite rise in counts—a phenomenon not observed in the euthyroid subjects.

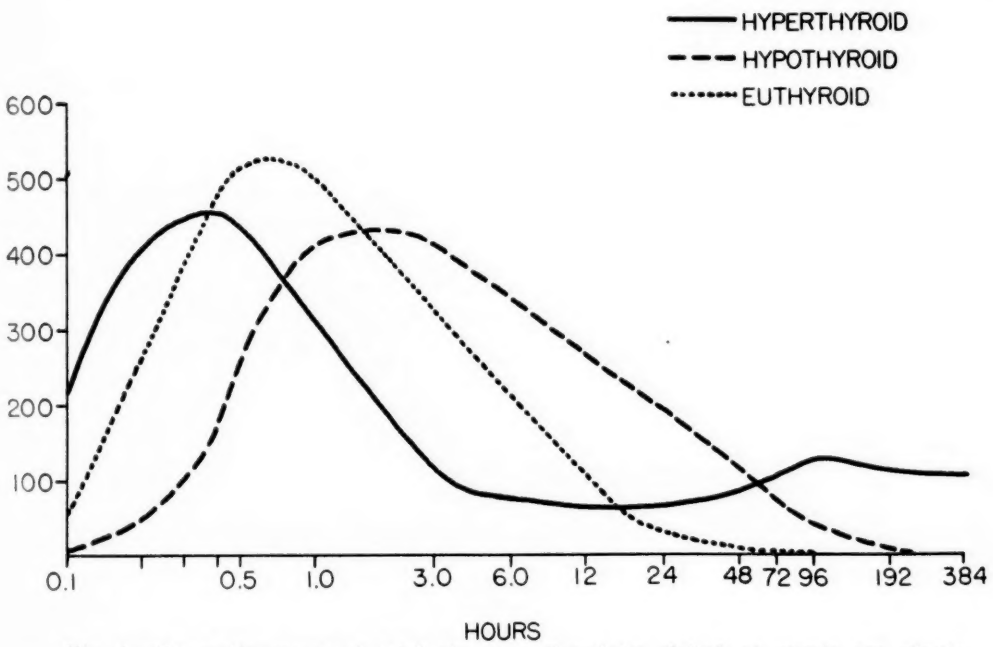

Fig. 1.—Composite curves of total plasma radioactivity plotted as counts per minute against time on a logarithmic scale of groups of hyperthyroid, hypothyroid, and euthyroid individuals.

Table V shows, in summary form, the results of I^{131} urinary excretion, thyroid pickup, and plasma curves; all three carried out on each patient in this study. For urinary excretion and thyroid pickup, the ranges in per cent are shown for each group and given times while the plasma levels are tabulated as to time of peak, time of low point and presence or absence of delayed rise of plasma radioactivity.

Urinary Excretion: This is where the greatest overlap existed between the three groups. There is indication that the increased output during the second twenty-four hours found in the myxedema cases deserves more attention. However, these patients were frequently not alert enough to collect their output reliably unless strictly supervised and the difference was not marked.

TABLE III. MYXEDEMA, SHOWING COUNTS PER MINUTE OBSERVED IN 0.2 C.C. PLASMA AT VARIOUS TIME INTERVALS AFTER INGESTION OF 100 MICROCURIES OF I^{131}

	TIME														
	MINUTES					HOURS								DAYS	
PATIENT	6	12	18	24	30	1	3	6	12	24	48	72	96	8	16
N. D.					75	212	373	333	307	237	121				
S. B.					88	345	381	358	385	325	205				
V. D.	19		193		158	223	296	252	199	116	31				
C. F.					347	541	541	291	213		125				
H. G.					270	429	665	532	358	252	127				
M. H.					493	500	400	325	261	142	80				
F. K.	2	36	87	138	307	440	374	268	245	172	64		18	16	0
M. L.					374	396	328	302	262	210	115	79	31	12	
J. L.					400	568	416	400	274	139	50			8	
G. M.					312	384		319	253	163					
M. O.					275	272	396	368	220	216	167				
B. R.					315	522	543	565	475	364	199	170			
M. V.					397	459	493	304	197	117					
Geometric mean of counts	6	36	130	138	309	396	423	346	271	188	102	116	24	12	

TABLE IV. HYPERTHYROIDS, SHOWING COUNTS PER MINUTE OBSERVED IN 0.2 C.C. PLASMA AT VARIOUS TIME INTERVALS AFTER INGESTION OF 100 MICROCURIES OF I^{131}

	TIME														
	MINUTES					HOURS								DAYS	
PATIENT	6	12	18	24	30	1	3	6	12	24	48	72	96	8	16
M. B.					813	941	580	289	136	57					
L. C.						495	321	225	198	183	218	158			
R. D.					255	163	71	39	40	53	64				
J. J.					555	400	258	63	18	16	48				
E. L.					280		148	125	148	184	195			84	
J. L.	203	477	417	495	417	325	90	48	56	85	156	158	154	158	143
A. M.			318		675	559	401	205	106	40	27				
G. M.					551	417	385	190	57	137	42			70	
G. T.	550	641	736	684	654	573	282	138	57	62	82	36	118	116	74
G. W.	101	232	396	341	328	211	35		56	78	131	156	153	152	124
M. K.	157	418	378	317	260	166	37	10							
Geometric mean of counts	205.1	415	447	437	441	320	109	89	71	70	86	109	141	110	113

Thyroid Pickup: This procedure proved valuable in the diagnosis of hyperthyroidism where a high count was reached in the first six hours, and continued as long as the first twenty-four hours. The ranges of counts of hypothyroid and euthyroid cases overlapped to an extent that made this an unsuitable tool for their diagnostic separation.

Plasma Curves: Here a considerable overlap between the euthyroid and hyperthyroid cases was present. Further counts during the first thirty minutes and after ninety-six hours may show a greater separation. The myxedema cases, however, were well separated by this method, showing a consistently slow rise, late peak, flat curve, and slow fall.

Two of the euthyroid "controls," both physicians, one male, one female, showed a low pickup, plus a high urinary excretion of I^{131}, and would have been classified as hypothyroid on that basis. Both of these were euthyroid

clinically and also with respect to protein-bound iodine and cholesterol levels; the plasma curves in both were euthyroid in configuration and concurred with the clinical impression.

Of note is the case of a third physician, not included in the composite groups, who had undergone a bronchogram with lipiodol over one year before he was studied with I^{131}. His protein-bound iodine was still too high to be measurable with the usual standards. His urinary excretion was 98 per cent in the first twenty-four hours. His thyroid pickup was 1.25 per cent and 2.5 per cent at six and twenty-four hours, respectively. His plasma levels followed the euthyroid configuration, however. The first sample taken at thirty-five minutes showed the highest count. The "low" was reached between twenty-four and forty-eight hours, and the eight-day count was zero.

TABLE V

TABLE "H"	HYPOTHYROID	EUTHYROID	HYPERTHYROID
Urinary excretion per cent	*Range*	*Range*	*Range*
First 24 hours	4.6-100	53-90	10-38
Second 24 hours	19-38	4-16	1-10
Total 48 hours	30-100	59-95	12-39
Pickup per cent	*Range*	*Range*	*Range*
6 hours	1-14	6-23	30-87
24 hours	1-15	9-38	51-94
48 hours	1-21	11-43	40-77
Plasma Curves			
Time of peak level	1-3 hours	30 minutes	30 minutes or less
Time of low point	72 hours	24-48 hours	12-24 hours
Late rise	No	No	Yes

DISCUSSION

In the periods following ingestion of carrier-free I^{131} at least five factors are operating, at times simultaneously, to influence the plasma level of radioactivity. These factors are: (1) absorption from the gut, (2) excretion into the urine, (3) trapping by the thyroid, (4) labeling of the bodies stable iodide, and (5) secretion into the blood stream of I^{131} labeled thyroid hormone.

The curves of plasma radioactivity may be divided into three time periods, as follows:

I. From ingestion of I^{131} to time of peak level of plasma radioactivity.

II. From peak level to time of disappearance or low point in level of plasma radioactivity.

III. Time after low point in radioactivity is reached.

The effect of the various factors described above on the plasma curves may then be considered during these three time intervals.

In period I, the rate of absorption by the hyperthyroid patient seems more rapid than in the other two groups as evidenced by the higher plasma level found in the earliest time period—six minutes—than in the euthyroid and hypothyroid groups. However, the much greater trapping ability of the hyperthyroid patient is evidenced by the lower level of plasma I^{131} attained at the peak in the hyperthyroid than euthyroid group. The slow rate of absorption,

coupled with a slow rate of urinary excretion, accounts for the curves seen with the myxedema patient. As evidence of this is the curve of patient T. B. R., the previously described euthyroid physician, who had an inability to trap I^{131} because of previous bronchograph. The plasma curve is that of the euthyroid individual, indicating that possibly the blood levels can be regulated to a degree by absorption and excretion rates independent of thyroid trapping.

The curves of the three groups of patients in period II, which has been arbitrarily selected as that time from the high point in the curve to its lowest point, represent the stage where the factors leading to an increment in plasma radioiodine are no longer dominant, and the twin factors of urinary excretion and possibly thyroid trapping are the dominant forces. The three curves are not parallel during this time. They become successively steeper, ranging from the slow fall of the myxedema patient to the very rapid drops seen in the euthyroid and hyperthyroid patients. The characteristics of the myxedema group during period II are probably governed almost entirely by the rate of renal excretion, inasmuch as there is very little clearing of plasma by means of thyroid trapping in these patients. The difference in slope between the euthyroid and hyperthyroid curves in this period is negligible, again suggesting that renal clearance rather than thyroid trapping accounts for the characteristics of the slope during period II. Whether this clearance is due to extra thyroidal factors rather than the thyroid activity itself is not clear.[1]

In period III the only patients showing change in radioactivity in the plasma were those in the hyperthyroid group. By this time the only important factor still operating is the reappearance of radioactivity as I^{131} bound to thyroid hormone. The reason for this rise in radioactivity as shown by the hyperthyroid patients is difficult to explain. A persistence or plateau of radioactivity residing within the "protein-bound" fraction of the plasma would be likely but the rise in activity after 48 hours would seem to indicate that there is at this time a rising rate of release of hormone from the thyroid which reaches its peak seventy-two to ninety-six hours after a given lot of iodine has been incorporated into hormone. The possibility that this rise merely represents accumulation of hormone secreted at a constant rate into the plasma cannot be disregarded.

SUMMARY

A comparison of the diagnostic usefulness of the twenty-four hour uptake of I^{131} by the thyroid, of the forty-eight hour urinary excretion of I^{131}, and of plasma levels of radioactivity was made on thirty-six patients (thirteen hypothyroid, eleven hyperthyroid, and twelve euthyroid). Both uptake and excretion studies showed a marked overlap between the euthyroid and hypothyroid patients.

Myxedematous patients had a slower rise in levels of plasma radioactivity and lost the radioactivity from the plasma more slowly than did nonmyxedematous patients. In the time periods studied the euthyroid and hyperthyroid patients were not clearly separated by plasma radioactivity measurements.

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ORGAN BLOOD VOLUME MEASUREMENTS IN NORMAL RATS

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IN TRACING the distribution of ingested or injected substances by extraction from individual organs, it is difficult to estimate what proportion of extracted material comes from the tissues and what proportion comes from the contained blood. When the relative weights of blood and tissue are unknown, precise quantitative studies on edema, hyperemia, or hyperplasia are not possible on the basis of organ weight changes and microscopic examination alone. Following the successful elaboration of a quantitative method for the extraction and measurement of a hematin derivative and T-1824 from tissues,[1] it became relatively simple to estimate the blood volume of an excised organ, as well as the ratio of erythrocytes to plasma within the organ. The purpose of this report is to present the results of such measurements on some selected organs and to consider their possible significance.

Methods for the determination of the blood volume of certain individual organs in vivo have been employed under somewhat specialized circumstances.[2-4] Essentially, these are based on a comparison of the dilution of some erythrocyte or plasma tag before and after the temporary occlusion of blood flow to the organ in question. Lagerlöf and his co-workers[5] have measured the blood volume of the heart and lungs on the basis of time-plasma concentration curves using T-1824 and cardiac catheterization techniques. In general, these in vivo methods, while entirely satisfactory for certain types of study, cannot be used for some of the same purposes as the post-mortem extraction methods. Measurements upon excised animal tissues have been made using radioiron tagged erythrocytes[6, 7] and hemoglobin extraction procedures.[8-11] Wish, Furth, and Storey,[12] employing radioactive tagged erythrocytes and tagged plasma, made measurements essentially similar to those reported here. If only organ blood and plasma volumes are needed, the dye-hematin method used here possesses no theoretical advantages over the radioactive method other than simplicity of technique and equipment. However, if these data are needed in tracing the organ distribution of some other radioactive tagged substance, the dye-hematin extraction method may eliminate some of the complexities in the required counting procedure.

The importance of organ blood volume measurements requires emphasis. There is an intimate relationship between the cells and the fluids which surround them. The factors which determine the qualitative and quantitative distribution of various substances between the cells and these fluids are highly

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complex. Diffusion, osmosis, filtration, storage, and metabolic conversion are some of the chief processes which account for this distribution. Most of the substances which play a role in cellular function are found in the blood stream at some time, and many of these are nearly always distributed between the blood and the cells. Specific studies based upon whole tissue extractions will usually measure not only the substance in the cells of the tissue but, in addition, they will also measure this substance as it exists in the blood which is trapped within that tissue. Therefore, it is apparent that a knowledge of the tissue blood volume and the concentration of some specific substance in that blood volume must be known in order to obtain a more accurate estimate of the true extravascular tissue content of that substance.

MATERIALS AND METHODS

Normal male rats of the Wistar strain were used. In most instances the serial sacrifice of animals is necessary to obtain data on the various organs. Each animal is lightly anesthetized with ether and restrained in the supine position on an animal board. A midline incision extending through the peritoneum is made from the symphysis pubis to the xiphoid cartilage.

Abdominal Viscera.—Loose-fitting cotton ligatures are placed around the renal artery and vein of each kidney at their organ junction. Ligatures are similarly placed around the porta hepatis. At zero time a known quantity of Evans Blue dye (about 30 mg.) is quickly injected into the inferior vena cava. After thirty seconds the ligatures are simultaneously drawn tight and one small clamp is placed on the subdiaphragmatic portion of the inferior vena cava and abdominal aorta while a second clamp is placed across the splenic pedicle. Thus the circulating blood volume in each organ under consideration is trapped. A rapid incision is made into the chest wall, and a blood sample is withdrawn directly from the heart for comparison and standardization. The liver, kidneys, and spleen are carefully removed from the animal with no loss of blood. After being weighed, each organ is homogenized and its Evans Blue dye content and hemoglobin content are determined by the method previously reported by this group.[1] Further observations show that, in this method, the ratio of extracted acid hematin derivative to the total hemoglobin content is constant.

Thoracic Viscera.—Thirty seconds after the injection of Evans Blue into the inferior vena cava, the anterior chest wall is rapidly incised bilaterally to provide a complete exposure of the heart and lungs. Clamps are immediately placed on the inferior vena cava as it enters the right heart and on the superior vena cava and ascending aorta as close to the heart as possible. This serves to trap the circulating blood volume of the heart and lungs as a unit. A standardizing blood sample is then withdrawn from a mesenteric blood vessel. The heart and lungs are removed and treated in the same manner described for the abdominal organs. Heart muscle myoglobin does not interfere in this determination of the hemoglobin content.

Blood Sample.—The blood sample from the heart or mesenteric vessel is transferred to a tube containing oxalate crystals. One portion of the blood is placed into a mercury calibrated Wintrobe tube and centrifuged for thirty minutes at 2,500 r.p.m. at a 15.2 cm. radius. The hematocrit is recorded and the dye-stained plasma is transferred to an optical cuvette where its Evans Blue dye content is measured at 610 mμ in a Beckman spectrophotometer. A second portion of the oxalated blood sample is withdrawn by a calibrated 20 cu. mm. hemoglobin pipette and allowed to hemolyze in 1.0 cubic centimeter of distilled water. This sample is then treated in the same manner as an organ homogenate so that a measurement of its Evans Blue and hemoglobin content can be determined.

We have observed that the exposed organs in the rat turn blue within five seconds after the dye is injected. Thirty seconds, therefore, seem sufficient for at least three complete circulations of the blood to occur. It is assumed that this allows for adequate mixing with the

TABLE I

RAT NO.	WEIGHT (GM.)	TOTAL BLOOD VOLUME (C.C.)	AVERAGE VENOUS HEMATOCRIT	RAT NO.	WEIGHT (GM.)	TOTAL BLOOD VOLUME (C.C.)	AVERAGE VENOUS HEMATOCRIT	RAT NO.	WEIGHT (GM.)	TOTAL BLOOD VOLUME (C.C.)	AVERAGE VENOUS HEMATOCRIT
2	450	41.5	0.505	10	280	19.2	0.452	20	425	23.4	0.481
3	200	15.7	0.471	11	390	33.5	0.556	21	400	31.5	0.447
4	225	17.3	0.503	12	505	58.0	0.676	22	330	26.0	0.443
5	300	23.3	0.501	13	375	30.5	0.540	23	355	29.1	0.439
6	475	49.3	0.570	14	515	36.8	0.492	24	320	26.1	0.466
7	435	33.6	0.536	15	430	31.9	0.481	25	360	30.9	0.434
8	320	22.5	0.454	16	445	31.6	0.490	26	225	17.2	0.477
9	300	21.5	0.437	17	450	35.6	0.450	27	225	24.4	0.427

TABLE II. BLOOD VOLUME DATA FOR KIDNEYS*

RAT NO.	ORGAN WEIGHT (GM.)	ORGAN HEMATOCRIT	OPV	ORBCV	OBV	PER CENT TBV	OBV/GM. TISSUE
6	3.41	0.420	0.477	0.345	0.822	1.66	0.241
7	4.74	0.436	0.543	0.420	0.963	2.86	0.203
8	2.79	0.347	0.518	0.275	0.793	3.52	0.284
9	2.98	0.329	0.647	0.317	0.964	4.47	0.323
10	3.02	0.377	0.827	0.502	1.33	6.93	0.441
11	3.20	0.366	0.675	0.388	1.06	3.16	0.332
12	4.38	0.517	0.567	0.610	1.18	2.03	0.269
13	3.26	0.297	0.649	0.274	0.923	3.02	0.283
17	3.48	0.298	0.787	0.334	1.12	3.16	0.322
20	4.62	0.391	0.586	0.377	0.963	4.11	0.208
21	3.38	0.252	0.534	0.180	0.714	2.26	0.211
22	2.72	0.313	0.385	0.175	0.560	2.15	0.206
23	3.71	0.472	0.525	0.470	0.995	3.42	0.268
24	2.31	0.295	0.481	0.201	0.682	2.61	0.295
Average							0.278
S. D.							0.0653 (23.5%)

*Represents left and right kidneys combined.
OPV—Organ plasma volume in cubic centimeters.
ORBCV—Organ red blood cell volume in cubic centimeters.
OBV—Total organ blood volume in cubic centimeters.
Per cent TBV—Per cent of the total body blood volume in the organ.

actively circulating blood without any appreciable loss of the dye from the circulation. Since a known quantity of dye is injected, the total plasma volume is calculated from the dye concentration in the blood sample. The total blood volume is then estimated on the basis of the average venous hematocrit. Analysis of the second portion of the blood sample by the extraction technique yields the quantity of extractable Evans Blue and acid hematin derivative per unit volume of blood from which the organ plasma and erythrocyte volumes are calculated.

RESULTS

The results are presented in Tables I to V. In comparing the deviation of the organ hematocrit from the average venous hematocrit within each organ group studied, we obtained a change of –26 per cent for the kidneys, –9 per cent for the liver, –9 per cent for the heart and lungs together, and a +21 per cent for the spleen. Whereas the per cent of the total blood volume found in each organ shows considerable variation, these studies indicate that, for a given organ, the organ blood volume per gram of tissue has a relatively constant value.

TABLE III. BLOOD VOLUME DATA FOR LIVER

RAT NO.	ORGAN WEIGHT (GM.)	ORGAN HEMATO-CRIT	OPV	ORBCV	OBV	PER CENT TBV	OBV/GM. TISSUE	
3	6.77	0.261	0.772	0.274	1.05	6.69	0.155	
4	6.31	0.305	0.676	0.297	0.973	5.62	0.154	
5	8.23	0.343	0.863	0.453	1.32	5.67	0.160	
14	17.1	0.478	1.41	1.30	2.72	7.39	0.159	
15	14.5	0.448	1.43	1.16	2.59	8.12	0.179	
16	15.6	0.460	1.28	1.09	2.37	7.50	0.152	
17	14.0	0.437	1.42	1.10	2.52	7.09	0.180	
20	12.8	0.545	0.955	1.15	2.11	9.03	0.165	
21	15.8	0.512	1.61	1.69	3.30	10.50	0.209	
22	13.6	0.535	1.14	1.31	2.45	9.43	0.180	
23	15.1	0.477	1.48	1.35	2.83	9.73	0.188	
24	13.1	0.358	1.35	0.755	2.11	8.09	0.161	
Average							0.178	
S. D.							0.0191	(10.70%)

TABLE IV. BLOOD VOLUME DATA FOR SPLEEN

RAT NO.	ORGAN WEIGHT (GM.)	ORGAN HEMATO-CRIT	OPV	ORBCV	OBV	PER CENT TBV	OBV/GM. TISSUE	
10	0.604	0.713	0.114	0.283	0.397	2.08	0.658	
11	0.788	0.627	0.157	0.264	0.421	1.26	0.535	
12	1.15	0.657	0.173	0.331	0.504	0.87	0.438	
13	1.19	0.748	0.127	0.377	0.504	1.65	0.423	
17	1.68	0.491	0.386	0.373	0.759	2.13	0.452	
20	0.860	0.501	0.216	0.217	0.433	1.85	0.504	
21	1.77	0.493	0.437	0.425	0.862	3.01	0.487	
22	1.19	0.562	0.208	0.267	0.475	1.83	0.400	
23	0.910	0.753	0.081	0.247	0.328	1.13	0.361	
24	1.07	0.459	0.260	0.221	0.481	1.84	0.450	
Average							0.481	
S. D.							0.0835	(17.36%)

TABLE V. BLOOD VOLUME DATA FOR HEART AND LUNGS

RAT NO.	ORGAN WEIGHT (GM.)	ORGAN HEMATO-CRIT	OPV	ORBCV	OBV	PER CENT TBV	OBV/GM. TISSUE
2	5.42	0.522	1.30	1.42	2.72	6.56	0.502
25	4.69	0.388	1.10	0.694	1.79	5.79	0.382
26	2.75	0.411	0.539	0.376	0.915	5.35	0.332
27	2.78	0.349	0.531	0.285	0.816	3.34	0.294
Average							0.379

DISCUSSION

Regardless of the type of tag used for either erythrocytes or plasma, certain intrinsic limitations of this procedure must be recognized and their potential effects on the final results must be considered. First, it is obvious that the volume of blood trapped in an organ by the procedure described above is at best only a reasonable approximation of that volume present prior to the experimental interference. Consistency in measurements of "organ blood volume" would depend chiefly on consistency of procedure. While this would be an important consideration where this method is employed as an index of change

in the distribution of circulating blood volume, precision of measurements per se could not be regarded as evidence of the true accuracy of post-mortem measurements.

A second consideration involves the assumption that the erythrocyte or plasma tag is uniformly distributed throughout the vascular compartments of the organ. Where a hemoglobin derivative is used as the index of erythrocyte content, uniformity of the mixing of tagged and untagged elements presents no difficulty. However, the use of plasma tags is somewhat more complex. Virtually every known plasma tag, regardless of molecular size, has a measurable rate of either escape from the plasma compartment into tissue spaces or uptake and concentration by reticuloendothelial elements. If sufficient time were allowed for complete equilibration of the plasma tag with all portions of the vascular compartments, the volume of plasma would be overestimated by an amount proportional to the loss of plasma tag from the vascular compartment. In considering measurements of total plasma volume in small animals, we have previously shown that the mixing time of dye with plasma is negligible.[13] Since the contribution of a given organ to the total plasma volume is relatively small, a prolonged local mixing time might not be appreciable in the total measurement. In the experiments reported here, the time interval between dye injection and separation of the organ was short. Therefore, the loss of dye from the plasma compartment was negligible. We realize that the plasma volume calculated on the basis of dye extraction may not include any relatively stagnant portions of the plasma compartment, but it clearly measures the volume in active circulation. It seems likely that the thirty seconds allowed for mixing in these studies is adequate and that the relatively stagnant plasma pools are negligible since measurements allowing five minutes for mixing[14] yield estimates of total blood volume of 8.15 ± 0.90 ml. per 100 Gm. Our estimate is 8.09 ± 0.27.

The deviation in the ratio of erythrocyte volume to plasma volume in viscera as compared to venous blood is consistent with the findings of other investigators.[15-17] Part of this deviation might be ascribed to the relatively greater penetration of plasma into those partially collapsed capillaries which impede the passage of erythrocytes. A more convincing explanation, however, is found in the studies of Fåhraeus.[18] He studied the behavior of the formed elements of the blood as they passed through glass capillary tubes and compared these findings with observations made upon vessels of varying caliber in the intact animal. Fåhraeus showed that the axial stream is composed of the largest formed elements with the progressively smaller ones being distributed between the axis and the vessel wall. As the vessel becomes smaller in diameter the relative velocity of the erythrocytes, as compared to the plasma, becomes greater so that at any instant the ratio of the volume of the plasma to the erythrocytes within the minute vessel becomes greater.

Regardless of the mechanisms involved in producing local distortions of the hematocrit, the fact that such distortions do exist accounts for the smaller estimates of total blood volume based on tagged erythrocytes. Such estimates

are calculated on the assumption that the peripheral venous hematocrit is equal to the mean hematocrit, but, as has been shown, the mean hematocrit is considerably smaller.

When the specific gravities of corpuscles and plasma are known, these extraction measurements can be used to determine the weight of the blood contained in tissues. When quantitative studies of pathological changes in organs are based upon weight changes, a correction for the weight of this contained blood will be useful. Moreover, when the distributions of radioisotopes or other blood-borne materials between the plasma and erythrocytes are known, these measurements can be used in correcting estimates of tissue uptake.

SUMMARY

By extracting Evans Blue and a hemoglobin derivative from homogenized tissues, using quantitative techniques, it is possible to measure organ blood volumes in the rat. Whereas the per cent of the total blood volume found in each organ shows considerable variation, the organ blood volume per gram of tissue is relatively constant for each respective organ. The average blood volume per gram of tissue in normal rats is 0.278 ml. for the kidney, 0.178 ml. for the liver, 0.481 ml. for the spleen, and 0.379 ml. for the heart and lungs combined.

The difference between the organ hematocrit and the average venous hematocrit was determined for each organ. These data are in general agreement with investigations of blood volume obtained by other methods and are not dependent upon the use of radioactive tracers. In quantitative studies which investigate the tissue distribution of blood-borne substances, a correction for the organ blood volume can be made.

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THE EFFECT OF L-NOR-ADRENALINE ON ELECTROLYTE EXCRETION IN NORMAL MAN

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IT IS well established that the renal excretion of electrolytes and water may be profoundly influenced by hormones of the adrenal cortex. Studies of the effects of adrenal medullary hormones on renal excretory function have been far less extensive and less conclusive. Furthermore, since adrenaline prepared from biological material contains about 85 per cent adrenaline and 15 per cent nor-adrenaline,[1] the results of investigations in which this material was employed are accordingly rendered uncertain, since these two hormones differ in their physiologic effects.

In the dog, adrenaline has been observed to increase urine flow,[2, 3] diminish it,[3, 4] or to produce no change.[5] Also in the dog, adrenaline has been reported to increase chloride excretion[3] and diminish sodium excretion.[5] In the rat, adrenaline has been noted to abolish pentothal-induced antidiuresis,[6] to augment diuresis,[7, 8] and to increase chloride excretion.[7] In man, "adrenin" has been reported[9] to produce oliguria. Depression of potassium excretion has also been noted.[10] In a study on four human beings,[11] the chronic administration of adrenaline has been reported to increase sodium and chloride excretion and to diminish potassium excretion. The adrenaline preparation therein employed contained nor-adrenaline as well. In a single patient with rheumatoid arthritis,[12] the chronic administration of adrenaline is reported to have produced increased sodium excretion and retention of potassium.

Studies on nor-adrenaline alone have been sparse. Eversole[13] reported the chloruretic action of "adrenaline" in rats to be due to its nor-adrenaline content. Drill and Bristol[14] have also observed diuresis and chloruresis in rats after nor-adrenaline administration. Horres and associates[15] have produced evidence that the diuretic action in rats of adrenal medullary substance is due more to nor-adrenaline than to adrenaline. In man[16] it is reported that nor-adrenaline infusion produced changes in urine flow that were "slight and probably not significant."

The present study was undertaken to determine the effect of chemically pure l-nor-adrenaline* on sodium, chloride, and potassium excretion in man, and to gain insight into the mechanisms by which such changes might occur.

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METHODS

Experimental Procedure.—The subjects were six clinically normal young men. Glomerular filtration rate was measured by inulin clearance, and effective renal plasma flow by p-aminohippurate (PAH) clearance. Each subject was placed on a normal diet of constant and known composition a week before the experiment was to take place. All studies were made in the morning after a ten-hour fast. After a suitable priming injection, a solution of inulin and PAH in 0.85 per cent saline was administered intravenously by a Bowman constant speed infusion pump at a rate calculated to produce an inulin concentration in serum of about 30 mg. per cent and a PAH concentration of 1 to 2 mg. per cent. Urine was collected by a multieyed catheter, and the bladder rinsed with distilled water or inflated with air at each emptying. In order further to minimize errors in urine collection, the studies were performed under water diuresis, each subject drinking 200 ml. of tap water every twenty minutes, starting two to three hours before the beginning of the experimental observations. Femoral arterial blood samples were collected at midperiod under oil by means of an indwelling Cournand needle. All blood was centrifuged and the serum separated with minimum possible delay.

After about thirty minutes (to allow for equilibration), a second slow intravenous infusion of 0.85 per cent saline was started in a different vein. The bladder was then emptied, and, after two control periods, a solution of 5 to 10 γ per milliliter of l-nor-adrenaline in 0.85 per cent saline was substituted for the control saline. The nor-adrenaline solution was also administered by constant speed infusion pump. Four successive pairs of clearance periods were then performed, stepwise increments in rate of infusion being made at the end of each pair of periods. The dosage range studied varied from 2.0 γ per minute to 18.3 γ per minute. The period of administration at each dosage rate was approximately twenty minutes.

Chemical Methods.—Inulin was determined in cadmium sulfate filtrates of serum and urine by the method of Roe.[17] PAH was determined on the same filtrates by the method of Smith and associates.[18] Chloride was determined by iodometric titration.[19] Sodium and potassium were analyzed by flame photometry employing the Perkin-Elmer internal standard flame photometer.

Calculations.—Inulin clearance was calculated in the conventional way as the product of the urine flow (ml. per minute) and the concentration of inulin in urine (mg. per 100 ml.) divided by the concentration in serum (mg. per 100 ml..) Clearance values for PAH and electrolytes were calculated in an analogous manner. All clearance values were corrected to a surface area of 1.73 M.[2] The excretions of the several electrolytes are expressed as "clearance" rather than the somewhat more customary "per cent filtered ion excreted" for reasons which will be apparent in another section. (See under "Discussion.")

All correlation coefficients, both simple and partial, are of the linear type, and were calculated according to standard methods to be found in most textbooks on statistics. Statistical significance of the correlation coefficients was estimated both by the "t-test" of Fisher[20] and by the "Z transformation" of Fisher.[20] (The results of these two tests proved to be quite similar.) A "P" value, derived either from the ratio of Z to its standard error or from the value of "t," of less than 0.05, indicates that chance alone could account for the observed correlation coefficient fewer than 5 times in 100. It is conventional to consider P values less than 0.05 as "significant," and those less than 0.01 as "highly significant." The values of P recorded in the text are derived from whichever statistical test imposed a more stringent criterion of significance in the particular case in question.

RESULTS

The results of an illustrative experiment are shown in Fig. 1. The glomerular filtration rate remained essentially unchanged, despite the fact that plasma flow diminished as the rate of nor-adrenaline infusion was increased. The clearances of sodium and chloride showed slight rises of doubtful significance toward the end of the experiment. (This was associated with a slight increase in urine flow. No particular trend was apparent in the sodium and chloride clearances of the six subjects as a group.) The largest change was observed

in the clearance of potassium, which fell sharply when the nor-adrenaline infusion was started, and continued to diminish as the rate of hormone administration was increased. The depression of potassium clearance seemed to parallel the depression of renal plasma flow more closely than it did the increase in dose of nor-adrenaline.

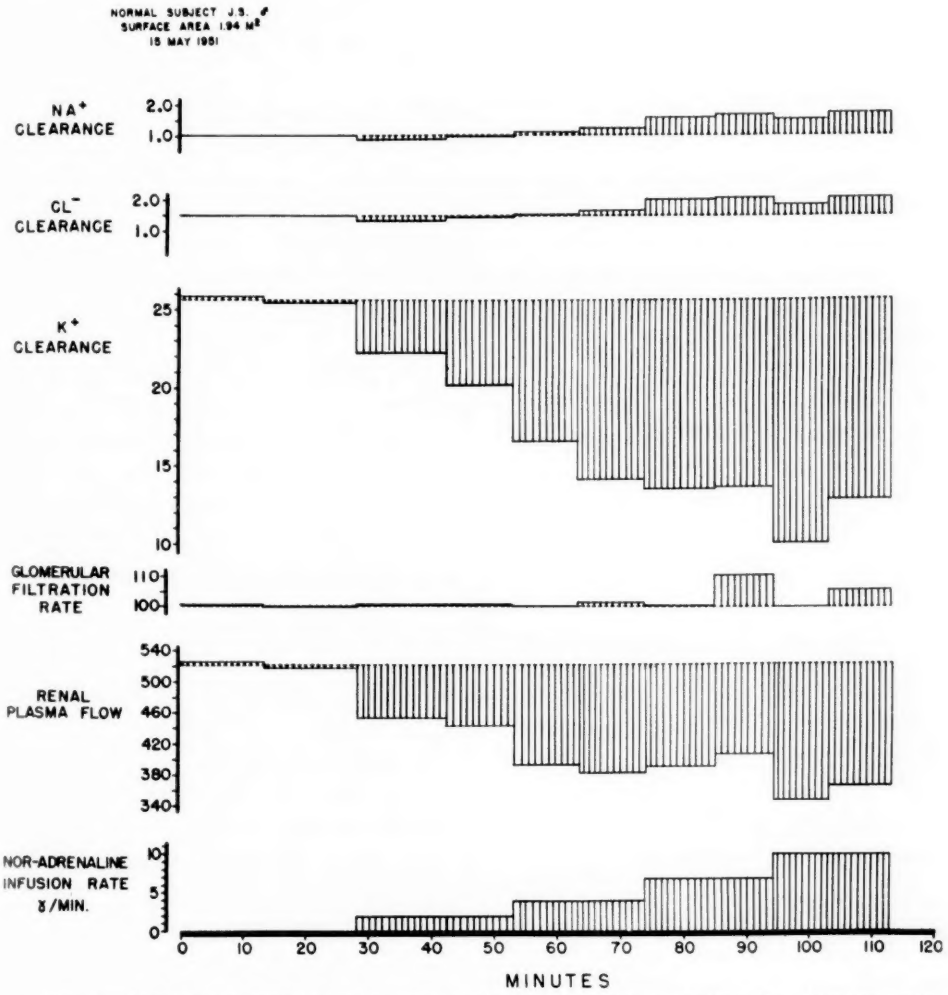

Fig. 1.—Illustrative experiment, normal subject. The bars indicating the filtration rate, plasma flow, and electrolyte clearances are erected from levels representing the mean values of these measurements during the control periods.

All six subjects reacted in essentially the same manner. Filtration rates remained unchanged. Clearances of sodium and chloride and urine flow showed small variations which appeared quite random. Potassium clearances (with one exception, at lower dose levels) fell with increasing nor-adrenaline dose. This fall in potassium clearance was due to a diminution in the excretory rate of potassium, and not to a fall in serum potassium. As a matter of fact, the serum potassium concentrations showed a small but statistically significant rise after nor-adrenaline was administered. This increase was evinced by all sub-

jects and averaged 5.2 per cent over control levels. It was not accompanied by statistically significant changes in serum sodium, chloride, or hematocrit values.

Fig. 2 illustrates the effect of nor-adrenaline dosage upon the potassium clearances of all six subjects. Despite the differences in control potassium clearances from individual to individual, all but one of the subjects evinced an unequivocal, fall in potassium clearance. In the single instance in which the potassium clearance rose, it did so only at the lower dosage ranges, and fell below control levels as higher nor-adrenaline doses were achieved.

DISCUSSION

No simple explanation suggests itself for the small but statistically significant rise in serum potassium that we have noted. It is not due to hemoconcentration because significant rises in sodium, chloride, and hematocrit did not occur. Since it has been shown that adrenaline administration induces a depression of plasma potassium,[10, 21, 22, 23] and that this is probably due to a shift of potassium from extracellular to intracellular fluid, one is tempted to speculate that the herein reported slight rises in serum potassium after nor-adrenaline administration might be due to migration of potassium from intracellular to extracellular fluid. A loss of potassium from the cells might conceivably be looked upon as a stimulus provoking the renal conservation of potassium as a homeostatic mechanism. On the other hand, the diminution in potassium excretion itself might contribute to the slight elevation of serum potassium.

In previous studies in which commercial "adrenaline" was used, it is difficult to separate the effects of adrenaline from those of nor-adrenaline. The fact that we have not observed with nor-adrenaline the chloruresis[11, 12] or natruresis[12] reported to occur in man after adrenaline administration is probably due to the fact that adrenaline and nor-adrenaline differ in their effects on electrolyte excretion. It may also be due, however, to differences in experimental design (the present study is not a "chronic" one) or to a possible masking effect of the water diuresis under which our observations were made. It is of interest to note that our studies on nor-adrenaline and also the previous ones on adrenaline have disclosed a fall in potassium excretion. It may prove fruitful to examine this change in light of the known facts about potassium excretion.

It has been shown[24-28] that under certain circumstances potassium is secreted by the renal tubules. It is not entirely clear whether this secretory mechanism is activated only upon the application of certain stimuli (e.g., cellular dehydration[25]) or whether it is in continuous operation, being overbalanced by the quantitatively greater rate of tubular reabsorption (thereby explaining the fact that the total excretory rate of potassium is, under normal circumstances, less than the rate of potassium filtration). Indeed, an experimental approach to this question is quite difficult to devise. Taking cognizance of these facts, the various possible mechanisms by which nor-adrenaline could depress potassium excretion may be outlined as follows:

1. Increased tubular reabsorption of potassium. (a) Directly as a result of nor-adrenaline augmenting the capacity of the reabsorptive potassium transport system. (b) Indirectly via other hormonal influences. (c) Kinetically, as a result of change in filtered potassium load.

2. Depression of tubular secretion of potassium. (a) Directly, as a result of nor-adrenaline depressing the capacity of the secretory potassium transport system. (b) Indirectly via other hormonal influences. (c) Kinetically, as a consequence of reduction of potassium load offered the tubules for secretion.

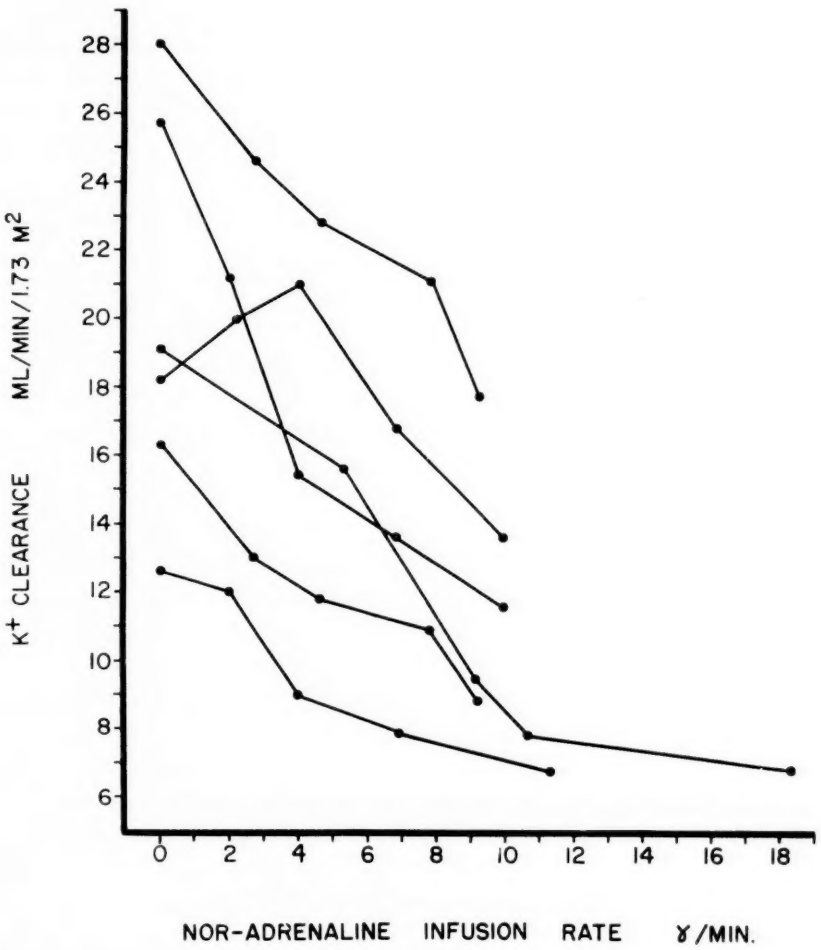

Fig. 2.—The effect of graded infusion rates of l-nor-adrenaline on potassium clearance in six normal men. Each dot represents the mean of two clearance periods during which the l-nor-adrenaline dose remained constant. The dots representing the various values observed in each subject are connected by straight lines.

Hypotheses 1(a), 1(b), 2(a), and 2(b) may be considered together and cannot be proved or disproved on the basis of available evidence.* Because adrenocortical activity is known to influence potassium excretion, and because adrenaline is thought to be a stimulus to the hypophyseo-adrenocortical system,

*The possibility exists that the observed depression of potassium excretion might be fortuitously related to nor-adrenaline dose, and actually be in some way related to the increased hydration of these subjects, all of whom were studied during water diuresis. We do not think this a likely possibility because the rate of urine flow showed no definite trend, nor did the potassium clearance correlate with urine flow.

it might be supposed that nor-adrenaline acts via a similar mechanism. However, since adrenocortical hyperactivity is associated with an increase rather than a decrease in potassium excretion, the assumption would also have to be made that nor-adrenaline, unlike adrenaline, depresses rather than stimulates the hypophyseo-adrenocortical system. There is no evidence for such an assumption.

Hypothesis 1(c) is untenable because the filtered load of potassium does not change greatly, since glomerular filtration rate remains constant and concentration of potassium in the glomerular filtrate does not diminish (but increases slightly instead). It is therefore apparent that nor-adrenaline exerts its influence upon potassium excretion via a tubular mechanism.

Hypothesis 2(c) could be subjected to experimental scrutiny by depressing the renal blood flow by drugs other than nor-adrenaline or by augmenting the renal blood flow. No data are at present available on the effect of the former of these procedures on potassium excretion. The latter (by induction of renal hyperemia with typhoid vaccine, the hyperpyrexia being suppressed by amidopyrine) has resulted in an augmentation of potassium excretion.[29] This finding is consistent with, but not proof of, hypothesis 2(c).

For secreted substances (below their maximal secretory rates) the rate of tubular secretion is a function of the total load offered the tubules (the product of the total volume of carrier [i.e., renal plasma flow] and the concentration of the particular substance in the carrier[30]). It would therefore be expected that as the potassium load diminishes (i.e., renal plasma flow decreases) the tubular secretion of potassium will decrease, and therefore the total potassium excretory rate will fall. Indeed, such does occur and the implications of this shall be examined quantitatively insofar as the data will allow.

Assuming that tubular secretion of potassium is continuous rather than intermittent and that both the reabsorptive and the secretory processes are operating at levels below their hypothetical maxima, it is possible to derive an expression defining the over-all excretory rate in terms of its hypothesized components.

Total excretory rate of potassium (E_K) will be a sum of the amount of potassium filtered per unit time, plus the amount added by the tubules per unit time (S_K) minus the amount reabsorbed by the tubules per unit time (t_K). The potassium filtered per unit time is given by the expression:

$$C_I \; P_K \; WD$$

where:

C_I = glomerular filtration rate (ml./min.)
P_K = concentration of potassium in plasma (mM/ml.)
W = correction factor relating the concentration of potassium in plasma water (i.e., glomerular fluid) to concentration of potassium in plasma.
D = correction for the effect of the Gibbs-Donnan equilibrium upon the distribution of potassium across the glomerular membrane.

We then have:

(1) $E_K = C_I \; P_K \; WD + S_K - t_K$

where: E_K = excretory rate of potassium (mM/min.)
S_K = tubular secretory rate of potassium (mM/min.)
t_K = tubular reabsorptive rate of potassium (mM/min.)

For many reabsorbed substances, the rate of reabsorption is proportional to the filtered load[30] (until a maximal rate of reabsorption is reached). Below this theoretical maximum, in the case of K +, we may set up, as a first approximation:

$$(2) \qquad t_K = a\, C_I\, P_K\, WD$$

where a is a proportionality constant. Similarly for a secreted substance, the rate of secretion is proportional to the load offered to the tubules.[30] The load of potassium delivered to the tubules may be calculated as the product of the volume of plasma perfusing the tubules and the concentration of potassium therein. However, any potassium filtered through the glomeruli and thereby diverted to the tubular lumina cannot properly be considered as part of the tubular load, and must be subtracted from the total load. This derivation is not vitiated by the possibility that tubular secretion of potassium is accomplished by an ion exchange mechanism.

$$(3) \qquad S_K = b\,(C_{PAH}\, P_K - WD\, C_I\, P_K) = b\, P_K\, (C_{PAH} - WD\, C_I)$$

where C_{PAH} is the effective renal plasma flow and b is a second proportionality constant.

Substituting these expressions for t_K and S_K into equation (1) we have:

$$(4) \qquad E_K = C_I\, P_K\, WD + b\, P_K\, (C_{PAH} - WD\, C_I) - a\, C_I\, P_K\, WD$$

and dividing by P_K

$$(5) \qquad E_K/P_K = WD\, C_I + b(C_{PAH} - WD\, C_I) - a\, C_I\, WD$$

The expression E_K/P_K is simply the "clearance" of potassium (C_K). Substituting:

$$(6) \qquad C_K = WD\, C_I - a\, WD\, C_I + b\, (C_{PAH} - WD\, C_I)$$

and simplifying:

$$C_K = WD\, C_I - a\, WD\, C_I - b\, WD\, C_I + b\, C_{PAH}$$

$$(7) \qquad C_K = C_I\, (WD - a\, WD - b\, WD) + b\, C_{PAH}$$

Since a, b, and D are constants, and since changes in W are relatively slight over short periods of time, we may consider the expression (WD – a WD – b WD) for practical purposes to be a constant and to represent this as A.

$$(8) \qquad C_K = A\, C_I + b\, C_{PAH}$$

This equation signifies that, if the assumptions underlying its derivation are correct, all other factors being equal, the potassium clearance should be functionally related to filtration rate and to renal plasma flow.

In the experiments herein reported, filtration rate remained essentially unchanged. Therefore, it would not be expected that potassium clearance should vary with glomerular filtration rate. However, renal plasma flow was reduced, often to 50 per cent of control values. The potassium clearance did parallel the plasma flow, as is illustrated in Fig. 1. However, an apparently equally tenable explanation for this observed relationship is that nor-adrenaline depresses both plasma flow and potassium clearance and that the latter two are therefore merely related by their mutual dependence upon a third variable.

In an attempt to determine which of the two related and simultaneously varying factors (i.e., C_{PAH} and nor-adrenaline dose) is of primary importance in depressing the potassium clearance, the technique of partial correlation may be employed. Simple correlation indicates a highly significant positive correlation between potassium clearance and plasma flow ($r = +0.741$, $P<0.001$) and a highly significant negative correlation between potassium clearance and

nor-adrenaline dose ($r = -0.618$, $P<0.001$). Partial correlation permits us to calculate the correlation of the first two variables, while holding the third variable constant statistically and thereby eliminating its influence. When this computation is carried out, the partial correlation coefficient between potassium clearance and plasma flow (holding nor-adrenaline dose constant statistically) is + 0.529 (still a highly significant value, $P = 0.01 - 0.001$), whereas the partial correlation coefficient between potassium clearance and nor-adrenaline dose (holding plasma flow constant statistically) is only − 0.118 (a value that cannot be proved significant, $P = 0.5 - 0.6$). These results are not materially changed when glomerular filtration rate is included with the other two variables with which potassium clearance is being partially correlated.

This relatively crude and indirect method indicates that the biologically significant relation is between potassium clearance and renal plasma flow. This would seem to favor hypothesis 2(c) above. However, caution must be exercised against drawing definitive conclusions via so inferential a technique. Before potassium clearance can be said to be functionally related to plasma flow (implying thereby the simultaneous tubular reabsorption and secretion of potassium), it would have to be demonstrated that other renal vasoconstrictors depress potassium clearance along with renal plasma flow, and further, that nor-adrenaline does not depress potassium clearance should the renal vasoconstriction be prevented by one of the benzodioxanes. The present data indicate the desirability of further investigation along this line, but by themselves cannot be considered to have established the functional significance of renal plasma flow as a factor modifying the potassium clearance.

SUMMARY AND CONCLUSIONS

The effect of graded infusions of l-nor-adrenaline upon electrolyte excretion has been studied in six normal men.

No apparent effects on chloride and sodium excretion were observed.

Potassium clearance was depressed.

The degree of depression of potassium clearance was more closely related to the concomitant depression of renal plasma flow than to the dosage of nor-adrenaline.

These findings are compatible with, but are not proof of, the hypothesis that reduction in renal plasma flow, by reducing the total tubular load of potassium, diminishes the tubular secretory rate of this ion.

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THE ERGOTHIONEINE CONTENT OF HUMAN ERYTHROCYTES; THE EFFECT OF AGE, RACE, MALIGNANCY, AND PREGNANCY

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THE development of improved methods for the determination of ergothioneine in biological fluids[1, 2] is facilitating investigations which may lead to an understanding of the physiological significance of this substance. Until recently our biological information about ergothioneine has been limited to the findings that it is present in ergot and in erythrocytes, that it probably occurs in elevated amounts in the blood of diabetics,[3, 4] and that there appeared to be a nutritional influence on its concentration in the blood of rats[5] and pigs.[6] Although there have been unsuccessful attempts to verify this nutritional influence,[7, 8] Spicer and his associates[9] have now presented convincing evidence for an effect of diet on the concentration of ergothioneine in rabbit erythrocytes. They have also shown that its concentration bears an inverse relationship to the rates of methemoglobin formation and reversion after treatment of the cells with sodium nitrite. Another observation of considerable significance is that of Leone and Mann,[10] who found that boar seminal vesicle secretion contains ergothioneine in concentrations several times higher than the blood of this species. Fraser,[11] using the diazo procedure, has confirmed the earlier reports of elevations in blood ergothioneine in diabetes mellitus. Furthermore, high concentrations were found in the blood of Indian and Eskimo patients with pulmonary tuberculosis, as compared to white patients with the same disease.[12] Although normal subjects of the former groups were not included in this study, it appeared that the differences were of racial rather than pathological origin.

When the bromine-labile sulfur method for the estimation of blood ergothioneine was first developed in this laboratory, there was no general agreement concerning the reliability of analytical methods previously reported. It therefore appeared desirable to employ this new method in a study of blood ergothioneine levels in man. Soon after the initiation of this work, the improved diazo procedure of Hunter was reported.[1] Since the two methods showed substantial agreement,[2] and since the diazo procedure is the more rapid one, the latter procedure was used in the remainder of the study. We have found that malignancy or pregnancy has little, if any, effect on red-cell ergothioneine content. The concentration in infants' blood averages somewhat lower than in adults, although we have confirmed Fraser's finding[11] that the age of adults has no influence on the results. The difference between infants and adults was found in both white and Negro subjects. Furthermore, in both age groups, higher average concentrations were found in blood from Negro subjects than from white.

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During our survey an observation of some interest was made concerning the diazo procedure. Hunter had reported that occasionally a 2 ml. aliquot of protein-free filtrate yields a proportionally lower value than a 1 ml. aliquot of the same solution. In such cases, he suggested the use of a 0.5 ml. aliquot, which, in his experience, usually yielded values in agreement with the 1 ml. portion. We encountered many extracts, particularly among Negro adults, which did not show this agreement. Indeed, the apparent blood ergothioneine was higher with the larger aliquots. We do not know the cause of this behavior, but it apparently is due to a substance whose enhancing effect is diminished with increasing dilution of the test solution.

METHODS AND MATERIALS

The analytical procedures of Hunter[1] and Touster[2] were used as described. The occasional bronze color described by Fraser[11, 12] was never observed in our experience with the former method, although infant blood occasionally gave an orange, rather than the usual violet-pink, solution. Fraser's modified procedure[12] did not eliminate this response. Moreover, we have observed that orange solutions occur frequently with extracts of low ergothioneine content.

Blood samples were obtained from patients in the Vanderbilt University Hospital and the Central State Hospital.* Unless otherwise noted, the subjects studied were at the former institution. The cancer group included some hospitalized patients, but most of this group were under care in the outpatient clinic. The infants were all attending well-baby clinic. The cases of pregnancy were uncomplicated.

RESULTS

Table I summarizes observations on the influence of the volume of protein-free extract used in the diazo test. The apparent ergothioneine content decreases with the volume of extract, and this effect is particularly noteworthy in analyses of blood with higher ergothioneine concentrations.

TABLE I. INFLUENCE OF VOLUME OF ALIQUOT OF PROTEIN-FREE FILTRATE IN HUNTER DIAZO METHOD, t TESTS FOR DIFFERENCES IN PAIRED VALUES

GROUP	NUMBER	ALIQUOT (ML.)	MEAN (MG. PER CENT ERG./ R.B.C.)	DIFF. ± S.E. OF DIFF. (MG. PER CENT ERG./R.B.C.)	PROB. LEVEL
A. White infants	7	0.5	7.9	0.13 ± 0.16	.45
		1.0	8.1		
B. White infants	21	1.0	5.7	0.42 ± 0.21	.06
		2.0	6.1		
C. Negro infants	8	1.0	10.5	0.88 ± 0.42	.07
		2.0	11.3		
D. Negro adults at C.S.H.	16	0.5	13.5	1.65 ± 0.38	$<.001$
		1.0	15.2	3.27 ± 0.41	$<<.001$
		2.0	18.5		

Table II summarizes the results obtained in the early part of the study, which was based on the bromine-labile sulfur method. Although the mean value for the uterine carcinoma group is somewhat larger than the others, the differences are not statistically significant. Further breakdown of the subjects

*Since this is a mental institution, it provided a means of obtaining blood samples from subjects under similar dietary influences. This point is particularly significant in a study of racial differences.

TABLE II. COMPARISON OF PREGNANT WOMEN, AND WOMEN WITH A HISTORY OF UTERINE CARCINOMA, WITH OTHER WHITE ADULTS (BROMINE-LABILE SULFUR METHOD)

GROUP	NUMBER	MEAN ± S.E. OF MEAN (MG. PER CENT ERG./R.B.C.)
A. Uterine carcinoma*	16	9.4 ± 1.1
B. Pregnant women	17	6.6 ± 1.0
C. Vanderbilt Univ. staff†	14	7.1 ± 0.9
D. Patients at C.S.H.‡	30	7.1 ± 0.8

*Comparison of this group with the university staff yields a t value of 1.60 (P = .12).

†Six males averaged 7.6 mg. per cent; eight females averaged 6.7 mg. per cent.

‡Three subgroups of ten subjects each, corresponding to ages 24 to 34 years, 45 to 56 years, and 58 to 75 years, averaged 7.9, 6.9, and 6.5 mg. per cent, respectively. The ten males and twenty females averaged 7.2 and 7.0 mg. per cent, respectively.

reported in this table disclosed no effect of age or sex, an observation in agreement with that of Fraser.[11] Division of the patients with malignancies into those with active or inactive disease, or of the pregnant women on the basis of trimester of pregnancy, did not yield noteworthy differences among such subgroups.

The data in Table II suggested further study of subjects with a history of uterine carcinoma. A series of patients with a history of nongynecologic neoplasms of various types was included in this new survey, in which the diazo method of analysis was employed. The former group averaged slightly higher than the latter, and than the university staff, but again the differences lacked statistical significance. These results are summarized in Table III.

TABLE III. EFFECT OF NEOPLASTIC DISEASE IN WHITE ADULTS (HUNTER METHOD, 2 ML. ALIQUOTS)

GROUP	NUMBER	MEAN ± S.E. OF MEAN (MG. PER CENT ERG./R.B.C.)
A. Uterine carcinoma*	15	11.2 ± 1.1
B. Nongynecologic neoplasms	19	9.4 ± 1.3
C. Vanderbilt Univ. staff	18	9.6 ± 0.6

*Comparison of this group with the university staff yields a t value of 1.36 (P = .20).

During the course of our investigation, it was observed that Negro patients with malignancies tended to have considerably higher red-cell ergothioneine concentrations than did the white patients. A study of this question of racial difference was therefore undertaken. At the same time, a number of analyses of the blood of infants was performed. The latter phase of the work became of added significance when Spicer observed a relationship between ergothioneine and nitrite-induced methemoglobin formation, since a number of cases of nitrite-induced methemoglobinemia in infants has been reported.[13]

Table IV summarizes the analytical work on the white and Negro groups. It is apparent that infants average considerably lower than adults, and that white subjects are lower than Negroes. A grouped distribution of individual values is given in Table V.

DISCUSSION

In view of Hunter's report[8] on the geographical variation in blood ergothioneine levels in certain species (rat, pig, human), it should be noted that the average value for normal white adults reported in the present paper is almost identical with that reported by Fraser[11] for Canadian subjects.* In

*The difference between average levels in a given group as determined by the two analytical methods employed in our study can be accounted for by inherent differences in these methods (see reference 2).

TABLE IV. COMPARISON OF WHITE AND NEGRO INFANTS AND ADULTS (HUNTER METHOD)*

GROUP	1.0 ML. ALIQUOT		2.0 ML. ALIQUOT	
	NUMBER	MEAN ± S.E. OF MEAN (MG. PER CENT ERG./R.B.C.)	NUMBER	MEAN ± S.E. OF MEAN (MG. PER CENT ERG./R.B.C.)
A. White infants	29	6.2 ± 1.0	21	6.1 ± 1.1
B. Negro infants	10	9.8 ± 1.4	8	11.3 ± 1.9
C. White Vand. Univ. staff	—	—	18	9.6 ± 0.6
D. White adults at C.S.H.	10	10.8 ± 0.8	—	—
E. Negro adults at C.S.H.	18	15.2 ± 1.2	18	18.5 ± 1.3

Significance tests for differences between groups

White vs. Negro: A vs. B (1 ml.), $t = 1.99$, $P = .06$
A vs. B (2 ml.), $t = 2.42$, $P = .02$
C vs. E (2 ml.), $t = 6.05$, $P = .001$
D vs. E (1 ml.), $t = 2.76$, $P = .01$

Infants vs. adults: A vs. C (2 ml.), $t = 2.55$, $P = .02$
A vs. D (1 ml.), $t = 2.68$, $P = .01$
B vs. E (1 ml.), $t = 2.99$, $P = .006$
B vs. E (2 ml.), $t = 3.30$, $P = .003$

Similar conclusions are reached by use of the chi square test.

*Where analyses are given for both 1 and 2 ml. aliquots for a group, the subjects upon which the 2 ml. results are based are included in the 1 ml. series. The double listing permits elimination of difference in analytical procedure in the comparison of different groups.

TABLE V. DISTRIBUTION OF ANALYSES SUMMARIZED IN TABLE IV

MG. PER CENT ERG./R.B.C.	1.0 ML. ALIQUOT				2.0 ML. ALIQUOT			
	INFANTS		ADULTS		INFANTS		ADULTS	
	WHITE	NEGRO	WHITE (C.S.H.)	NEGRO (C.S.H.)	WHITE	NEGRO	WHITE (VAND.)	NEGRO (C.S.H.)
<3	8	0	0	0	6	0	0	0
3-5.9	12	0	0	0	8	0	1	0
6-8.9	4	6	3	0	4	4	6	0
9-11.9	3	1	4	5	1	1	8	1
12-14.9	0	1	3	6	1	1	3	6
>15	2	2	0	7	1	2	0	11
Total	29	10	10	18	21	8	18	18

regard to racial differences, the latter investigator is of the opinion that diet was of little significance in explaining the elevated values found for Indians as compared to white patients.[12] We have a similar view, inasmuch as our white and Negro Central State Hospital groups had similar diets.

Spicer's work[9] on the relationship between ergothioneine and methemoglobin may supply the most promising clue concerning the physiological role of ergothioneine. Methemoglobinemia from ingestion of nitrate-contaminated well water has been found only in very young white infants.[13] The low ergothioneine values for white infants are consistent with the sensitivity of this group to nitrite-induced methemoglobinemia.* It is of course possible that these results are unrelated, and it is hoped that further work will disclose the extent of the relationship between them.

Further investigation of the role of diet in the formation of blood ergothioneine is also desirable. Research on the physiology of this substance will undoubtedly be facilitated by the synthesis of ergothioneine,[14] which will permit the preparation of isotopically-labelled material for biological study, and also by a new paper chromatographic analytical procedure.[15] In this regard, it

*Nitrate is apparently reduced to nitrite by intestinal bacteria before absorption.

may be worth noting that useful, reliable facts about ergothioneine have not been readily obtained in the past and that research on this substance should be considered quite critically. Most analyses at the present time are based upon Hunter's improved diazo procedure. We have found, however, that even when this method and the bromine-labile sulfur method indicate the presence of a considerable amount of ergothioneine in a tissue extract, the substance may not be detectable by paper chromatography. This disagreement may perhaps be explained by the occurrence of ergothioneine conjugates. Another illustration of a limitation in analytical technique was discussed in this paper, namely, a dependence of apparent ergothioneine concentration on the size of the aliquot of protein-free filtrate used in the diazo procedure.

SUMMARY

The diazo and bromine-labile sulfur procedures have been used in a study of ergothioneine blood levels in human beings. Infants average considerably lower than adults, as do white subjects in comparison with Negroes. Pregnant women and patients with neoplastic disease were not found to vary significantly from normal, although there appeared to be a tendency for women with a history of uterine carcinoma to have slightly higher values. A source of error in the diazo procedure has been reported.

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FATE OF CHOLINE IN RATS WITH AND WITHOUT EXPERIMENTAL HEPATIC INJURY

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THE metabolism of choline, to which an important role in the transport of fat from the liver[1] is ascribed, has attracted great interest in the last few years. Recently it was found that intestinal bacteria play an important part in the utilization of choline normally and that in liver diseases the fate of choline in the intestine is changed.[2] It can be demonstrated that the normal human being excretes about 60 per cent (expressed in terms of nitrogen) of orally administered choline in the urine in twenty-four hours as trimethylamine and its oxide. That this transformation of choline is carried out by intestinal bacteria is evidenced by several observations: (1) the urinary trimethylamine† excretion is reduced after suppression of intestinal bacteria by antimicrobial agents; (2) it is also reduced if the utilization of choline by the bacteria is diminished by feeding carbohydrates, and (3) intravenous administration of choline fails to increase significantly the urinary trimethylamine excretion.[3] The urinary excretion of trimethylamine after ingestion of choline is markedly reduced in patients with liver disease; prolonged treatment with high doses of choline raises it to normal simultaneously with a relative increase of the choline-splitting bacteria.[4] The altered fate of choline in liver diseases required further elucidation. Clarification of some of the problems raised is difficult in the human being. Therefore, it appeared desirable to perform animal experiments despite the probable differences in the alimentary tract between rat and man. Rats are known to transform choline to trimethylamine.[5] If this amount is comparable to that in the human being, a simple procedure is available to demonstrate the site of the choline transformation in the intestine and to compare, by investigation of the feces, the efficiency of choline absorption with the urinary trimethylamine excretion. Based on this information, it would be possible to study factors which influence intestinal utilization and absorption of choline, such as its administration in bound form (as lecithin) or combined with fat or with drugs influencing intestinal motility. Furthermore, it would be possible to study the effect of acute and chronic liver damage as well as of choline deficiency upon the utilization of choline.

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†Trimethylamine in the following refers to both trimethylamine and its oxide, since the method used does not separate the two.

MATERIAL AND METHODS

Wistar strain white rats weighing 150 to 200 Gm. were used. To produce acute hepatic changes, carbon tetrachloride (0.03 ml. per 100 Gm. body weight), bromobenzene (0.05 ml. per 100 Gm. body weight), and ethionine (100 mg. per 100 Gm. body weight) were given intraperitoneally, and the trimethylamine excretion was measured twenty-four to seventy-two hours after their administration. Bromobenzene was given after sixteen hours of fasting. For ethionine, only female rats were used.

Several procedures were used to produce chronic liver damage. After the periods of exposure listed below for the individual intoxications, the urinary excretion of trimethylamine after oral administration of a test dose of choline was measured. To a basic synthetic diet containing, in addition to essential salts and vitamins, 15 per cent casein, 76 per cent sucrose and 5 per cent corn oil, (a) 5 per cent carbon tetrachloride, (b) 0.5 per cent ethionine, and (c) 5 per cent bromobenzene was added. The rats receiving carbon tetrachloride and bromobenzene were killed after eight weeks on the diet, those receiving ethionine after five weeks. The other rats were fed a synthetic diet of vitamins, salt supplements, 12 per cent casein, 5 per cent corn oil, and 79 per cent sucrose plus 0.6 per cent butter yellow (paradimethylaminoazobenzene)[6] for three months. Another group of rats was kept for four weeks on a high-fat, low-protein diet-containing supplements, 6 per cent casein, 38 per cent hydrogenated cottonseed oil, 5 per cent cod liver oil, and 49 per cent sucrose.

To produce choline deficiency, rats were kept for five weeks on a diet containing vitamin and mineral supplements, 20 per cent alcohol extracted peanut meal, 6 per cent casein, 65 per cent dextrose, 3 per cent corn oil and 2 per cent cod liver oil. The peanut meal used contained 0.61 mg. choline base per gram.

In another group of rats, the trimethylamine excretion after choline intake was determined 144 to 192 hours after ligation of the common duct.

In a control series, urine of fasting untreated animals was collected in metabolic cages. The experimental rats received the test dose of choline chloride by stomach tube or intraperitoneally, were kept fasting in individual metabolic cages, and the urine was collected for forty-eight hours under 3 ml. of 5N hydrochloric acid. The urine samples were made up to 30 milliliters. After addition of enough 10N hydrochloric acid to make the final concentration 1N, about 1 Gm. of Devarda's alloy was added and the urines kept in a water bath at 85° to 95° C. for forty minutes. After cooling to room temperature, the samples were centrifuged and the supernatant fluid collected. The residue was washed with 5 ml. of water which were added to the previously obtained supernatant. The mixture was neutralized with saturated sodium hydroxide and 8 ml. of saturated potassium carbonate were added. The samples were then aerated for 2 hours and the trimethylamine collected in 0.5N hydrochloric acid. This was then treated with 3 per cent Reinecke salt solution. The crystalline precipitate developing within two to three hours was spun down and washed with aqueous saturated solution of trimethylamine Reineckate. The precipitate was then dissolved in acetone and the red color read in a spectrophotometer at 520 millimicron, using acetone as the blank. The readings were interpolated with a standard curve made by treating a standard solution of trimethylamine hydrochloride in a similar manner. The results were expressed as trimethylamine nitrogen. Choline was determined in urines alkalinized to thymolphthaleine, filtered and subsequently treated with Reinecke salt solution, following the method previously described.[2] Both trimethylamine and choline were identified in the urine as described before.[2, 3]

For the determination of choline in the stools, the feces were extracted with 50 ml. of ethyl alcohol. After filtration the alcohol was concentrated by heating to about 2 ml., then was evaporated to dryness at 37° C. The residue was extracted with 5 ml. of 1N hydrochloric acid to which 0.3 Gm. of Norit A was added. To 3 ml. of the filtrate, 2 ml. of Reinecke salt solution were added and the mixture kept overnight in the refrigerator. If a crystalline Reineckate formed, it was centrifuged and washed with saturated solution of choline Reineckate in 0.1N sodium hydroxide.

RESULTS

Normal Rats.—Rats on a stock diet which had received a test dose of 7.5 to 30 mg. choline chloride by stomach tube excreted in the urine approximately 40 to 46 per cent of this amount as trimethylamine (expressed in nitrogen) within 48 hours. Control animals (not receiving a test dose) excreted very small amounts of trimethylamine during such a period. No choline could be found in the stools. After intraperitoneal administration of 30 mg. choline, the urinary trimethylamine excretion did not rise above that in the control period (Table I). The urinary excretion of choline did not exceed 1 per cent of the administered dose after oral intake of 30 mg. of choline, whereas after intraperitoneal administration of the same amount, 6.8 to 9.2 per cent of the choline was found in the urine (Table II).

Site of Choline Transformation.—The contents of the gastrointestinal tract of rats immediately after sacrifice were obtained under sterile conditions by scraping the mucosa with a bacteriologic loop. Ten cubic centimeters of a tryptose medium containing 100 mg. choline chloride were inoculated with the contents and incubated for twenty-four hours at 37° C. The inoculum obtained from stomach and almost the entire small and large intestines caused transformation of a considerable amount of the choline in the culture medium to trimethylamine. Only the inoculum from a small strip corresponding to a part of the

TABLE I. URINARY EXCRETION OF TRIMETHYLAMINE OF NORMAL RATS WITHIN FORTY-EIGHT HOURS AFTER INTAKE OF CHOLINE CHLORIDE

NO. OF RATS	AMOUNT OF CHOLINE CHLORIDE IN MG.	ROUTE	TRIMETHYLAMINE EXCRETED WITHIN 48 HOURS IN MG. NITROGEN		% TRIMETHYLAMIN NITROGEN FROM CHOLINE NITROGEN ADMINISTERED		CHOLINE IN FECES	
			MEAN	RANGE	MEAN	RANGE	NO. OF RATS	RESULTS
6	–	–	0.076	0.065-0.085	–	–	–	–
5	7.5	oral	0.330	0.270-0.395	44.0	36.0-52.4	–	–
14	15.0	oral	0.612	0.510-0.700	40.5	34.0-46.8	7	Negative
7	30.0	oral	1.380	1.180-1.520	46.2	39.4-50.7	4	Negative
4	30.0	intraperitoneal	0.075	0.060-0.085	0.25	0.20-0.27	2	Negative

TABLE II. URINARY EXCRETION OF CHOLINE OF NORMAL RATS WITHIN FORTY-EIGHT HOURS AFTER INTAKE OF 3 MG. OF CHOLINE CHLORIDE NITROGEN (CORRESPONDING TO 30 MG. OF THE SALT)

ROUTE	CHOLINE EXCRETED IN MG. NITROGEN
Oral	0.025
Oral	0.030
Oral	0.020
Oral	0.031
Oral	0.035
Oral	0.019
Intraperitoneally	0.210
Intraperitoneally	0.240
Intraperitoneally	0.260
Intraperitoneally	0.250
Intraperitoneally	0.225
Intraperitoneally	0.275
Intraperitoneally	0.235
Intraperitoneally	0.205

small intestine following the entrance of the common duct into the duodenum failed to transform choline (Fig. 1). Media inoculated from this portion proved to be sterile also by routine bacteriologic methods.

Combination of Choline with Lipids.—A much smaller percentage of choline (expressed on nitrogen basis) if given as lecithin was excreted into the urine as trimethylamine. If choline was administered together with corn oil, the trimethylamine excretion was also considerably lower. Similarly, administration of choline combined with Tween 60 (Polyoxyethylene sorbitan monostearate)* reduced the trimethylamine excretion. It was especially low in the rats in which the administration of Tween 60 led to loose diarrheic stools. These were the only instances in which the stool gave a positive choline reaction (Table III). Trimethylamine was never demonstrated in the feces.

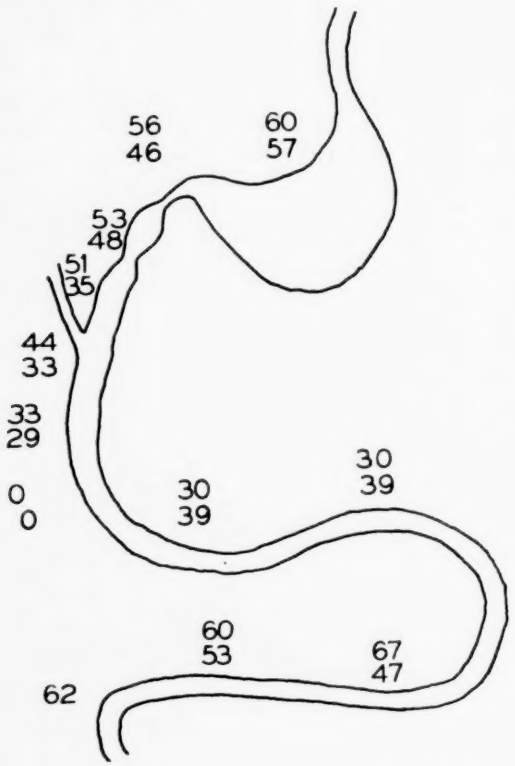


Fig. 1.—Percentage of choline transformed to trimethylamine in a choline containing culture medium inoculated with the content of different levels of stomach and small intestine.

Alteration of Intestinal Motility.—The doses of atropine or prostigmine administered influence intestinal motility and have been shown to reduce absorption of vitamin A (as in the case of atropine) or to increase it (as in the case of prostigmine).[8] Nevertheless their administration was without any effect upon the trimethylamine excretion after choline intake, and did not lead to fecal choline excretion (Table III).

*Obtained through the courtesy of Dr. J. P. Kass of the Atlas Powder Company, Wilmington, Del.

Hepatic Damage.—In rats with acute carbon tetrachloride intoxication, extensive necrosis in the central portion of the lobule and hydropic cells, and marked fatty metamorphosis in the intermediate portion was noted; the

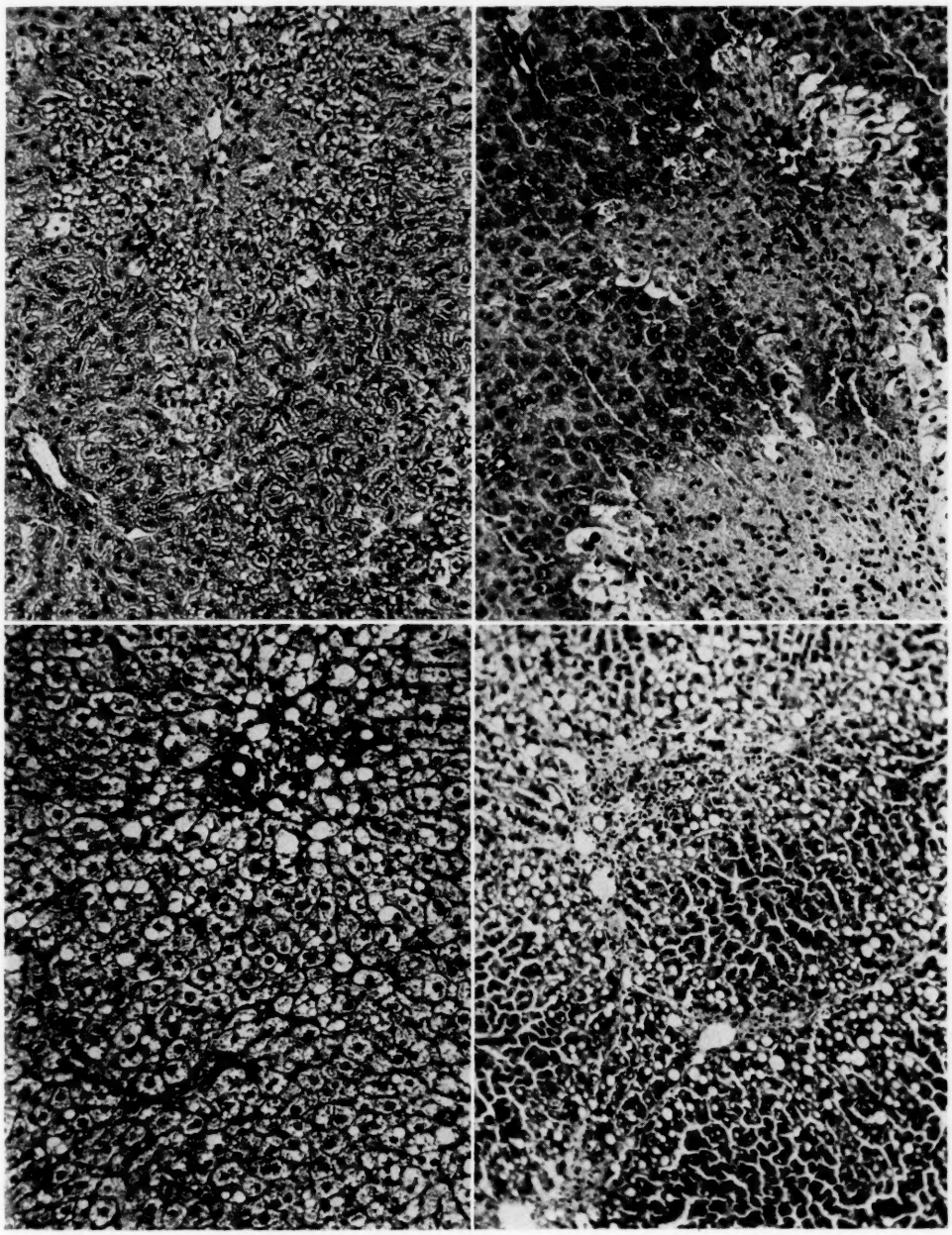


Fig. 2.—Hematoxylin-eosin-stained paraffin sections of rat livers. (×80.) *A*, Acute carbon tetrachloride intoxication. Necrosis and disappearance of liver cells in the centrolobular zone. Hydropic swelling and fatty metamorphosis in the intermediate zone. *B*, Acute bromobenzene intoxication. Eosinophilic necrosis of liver cells and proliferation of histiocytes in centrolobular zone. Hydropic degeneration of liver cells bordering the necrosis. *C*, Acute ethionine intoxication. Diffuse fatty metamorphosis without necrosis. *D*, Chronic carbon tetrachloride intoxication. Distortion of the lobular pattern by connective tissue septa connecting the central fields. Focal necroses and fatty metamorphosis.

TABLE III. URINARY TRIMETHYLAMINE EXCRETION (EXPRESSED IN PER CENT OF CHOLINE NITROGEN ADMINISTERED) OF RATS AFTER INTAKE OF CHOLINE. THE RATS RECEIVED SUPPLEMENTS WITH THE CHOLINE OR WERE TREATED PRECEDING CHOLINE ADMINISTRATION.

NO. OF RATS	TREATMENT OR SUPPLEMENT	CHOLINE CHLORIDE (MG.)	TRIMETHYLAMINE EXCRETION		CHOLINE IN FECES	
			MEAN	RANGE	NO. OF RATS	RESULTS
6	Lecithin, 300 mg. (containing 34.5 mg. choline chloride)	0	23.7	20.2-27.6	6	Neg.
8	Corn oil, 1 ml.	30	20.4	14.2-27.0	8	Neg.
5	Tween 60, 1 Gm.	30	19.8	16.6-22.6	5‡	Pos.
4	Tween 60, 1 Gm.	30	23.5	22.3-27.0	4	Neg.
3	Atropine*	15	42.5	40.0-45.3	3	Neg.
	Prostigmine†	15	41.4	38.6-44.6	3	Neg.

*0.1 mg. per 100 Gm. given twice, thirty minutes before and thirty minutes after the choline administration.

†0.2 mg. per 100 Gm. given twice, thirty minutes before and thirty minutes after the choline administration.

‡Loose diarrheic stools.

TABLE IV. URINARY TRIMETHYLAMINE EXCRETION (EXPRESSED IN PER CENT OF CHOLINE NITROGEN ADMINISTERED) OF RATS AFTER INTAKE OF 15 MG. CHOLINE CHLORIDE. THE RATS HAD DIFFERENT TYPES OF ACUTE OR CHRONIC HEPATIC INJURY

NO. OF RATS	INTOXICATION	MEAN FAT IN 100 GRAMS LIVER		DEGREE OF LIVER CELL DAMAGE	TRIMETHYLAMINE EXCRETION		CHOLINE IN FECES
		MEAN	RANGE		MEAN	RANGE	
4	Acute CCl_4	13.2	10.2-16.4	++	37.8	35.5-40.0	Negative
6	Acute bromobenzene	5.8	5.2- 6.1	+++	39.0	36.5-43.0	Negative
3	Acute ethionine	24.9	20.2-30.9	±	38.2	37.0-40.0	Negative
4	Chronic CCl_4	17.0	12.0-20.0	++ – +++	21.3	14.8-25.5	Negative
8	Chronic ethionine	3.3	2.5- 4.2	+++	12.3	8.3-17.0	Negative
6	Chronic bromobenzene	7.3	6.9- 7.8	++ – +++	28.3	24.2-41.0	Negative
9	Butter yellow	7.2	6.3- 8.9	+ – ++	23.9	16.2-28.4	Negative
4	Common duct ligation	6.2	5.5- 6.8	+	36.9	34.0-39.4	Negative
5	Choline deficient	19.6	18.6-21.6	–	36.2	31.5-42.0	Negative

TABLE V. URINARY TRIMETHYLAMINE (EXPRESSED IN PER CENT OF CHOLINE NITROGEN ADMINISTERED) OF RATS AFTER ORAL INTAKE OF 15 MG. CHOLINE CHLORIDE. THE RATS HAD RECEIVED A HIGH-FAT LOW-PROTEIN DIET FOR 4 WEEKS WITH VARIOUS SINGLE OR CONTINUOUS SUPPLEMENTS.

NO. OF RATS	SUPPLEMENT	FAT IN LIVER TISSUE (GM. PER CENT)		DEGREE OF LIVER CELL DAMAGE	TRIMETHYLAMINE EXCRETION		CHOLINE IN FECES
		MEAN	RANGE		MEAN	RANGE	
12	–	21.0	15.2-28.0	++	24.3	16.8-34.0	Negative
4	60 mg. choline chlor.*	16.4	12.0-21.1	++	25.5	22.5-42.0	Negative
3	15 mg. choline chlor.†	15.8	13.3-20.0	++	29.7	28.0-30.5	Negative
8	30 mg. choline chlor.‡	9.3	7.3-12.4	++	26.0	18.2-31.2	Negative
3	500 mg. choline per kilogram diet	20.6	19.8-22.0	++	22.7	20.7-25.0	Negative
4	5 per cent bromobenzene in diet	7.6	6.6- 8.7	+++	17.4	11.7-21.0	Negative

*Intraperitoneally in three daily doses from two to five days before administration of the test dose.

†Intraperitoneally two days before administration of the test dose.

‡Fifteen milligrams orally on the second and third day before administration of the test dose.

peripheral zone was normal (Fig. 2, *A*). Acute bromobenzene intoxication[9] produced extensive central necrosis with hydropic degeneration of, but without fat deposition in, the surrounding cells (9) (Fig. 2, *B*). In both conditions the

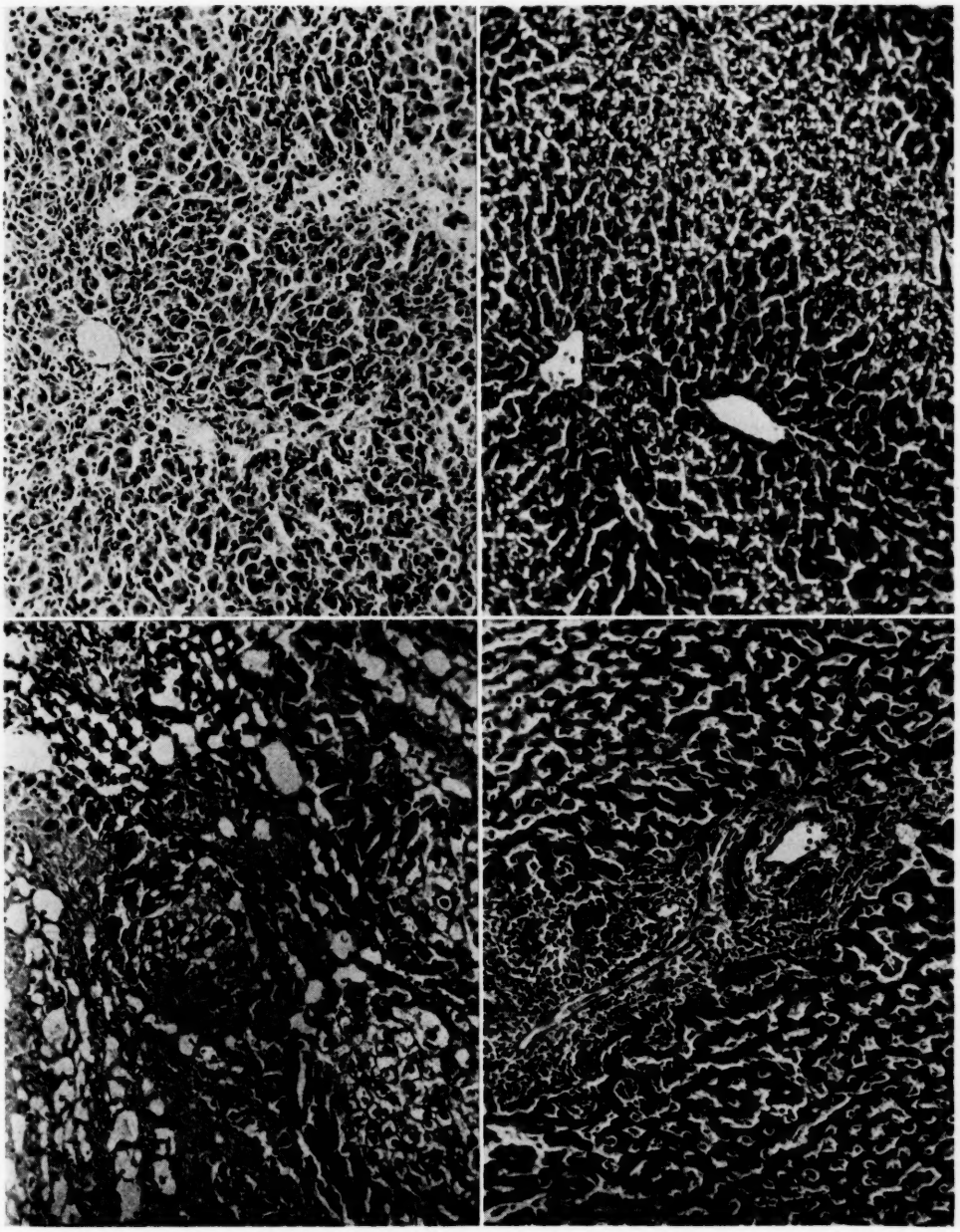


Fig. 3.—Hematoxylin-eosin-stained paraffin sections of rat livers (×80.) *A*, Chronic ethionine intoxication. Degeneration, necrosis and attempt at regeneration of liver cells, which vary markedly in size and staining qualities. Single cells and small groups of cells are surrounded by strands of connective tissue of variable width containing histiocytes and proliferated cholangioles. *B*, Chronic bromobenzene intoxication. Hydropic degeneration and clumping of cytoplasm of liver cells chiefly in the peripheral zone, where the liver cell plates are irregularly arranged. *C*, Chronic butter yellow intoxication. Necrobiosis, necrosis and irregular regeneration of liver cells, some of them arranged in nodules. Irregular proliferation of cholangioles of adenomatous character. *D*, Common duct ligation. Proliferation of bile ducts and cellular infiltration in portal triads. Bile stasis, Kupffer cell mobilization and slight liver cell damage.

trimethylamine excretion after choline was normal or almost normal despite a marked liver cell damage. Ethionine, a presumed antagonist of methionine, produced diffuse fatty metamorphosis of the liver without significant necrosis in female but not in male rats[10, 11] (Fig. 2, *C*). With this acute fatty liver, the trimethylamine excretion after choline was also not significantly altered. The liver of rats with the chronic carbon tetrachloride intoxication showed moderate fatty metamorphosis and some necrosis of individual liver cells associated with distortion of the lobular pattern characterized by connective tissue septa connecting the central field and occasionally the portal canals (Fig. 2, *D*). Prolonged ethionine administration caused marked damage of individual liver cells as well as extensive intralobular fibrosis which separated individual liver cells. Proliferation of fibroblasts and cholangioles was also noted[12] (Fig. 3, *A*). In chronic bromobenzene intoxication the liver cells appeared damaged, especially in the peripheral zone and dissection of the lobular pattern by septa originating from the portal triads was noted (Fig. 3, *B*). In chronic butter yellow intoxication marked dissection of the lobular pattern, necrosis of groups of liver cells, and proliferation of cholangioles, in some areas resembling adenomas, was noted (Fig. 3, *C*). In these four types of chronic hepatic injury, in contrast to acute injury, the trimethylamine excretion was significantly reduced. The reduction was most marked in chronic ethionine intoxication in which the liver damage was the most severe. In general, the reduction of the trimethylamine excretion paralleled the degree of liver cell damage in chronic hepatic injury (Table IV). Ligation of the common duct which resulted in biliary stasis, proliferation of bile ducts, and some liver cell damage (Fig. 3, *D*) failed to alter the trimethylamine excretion significantly.

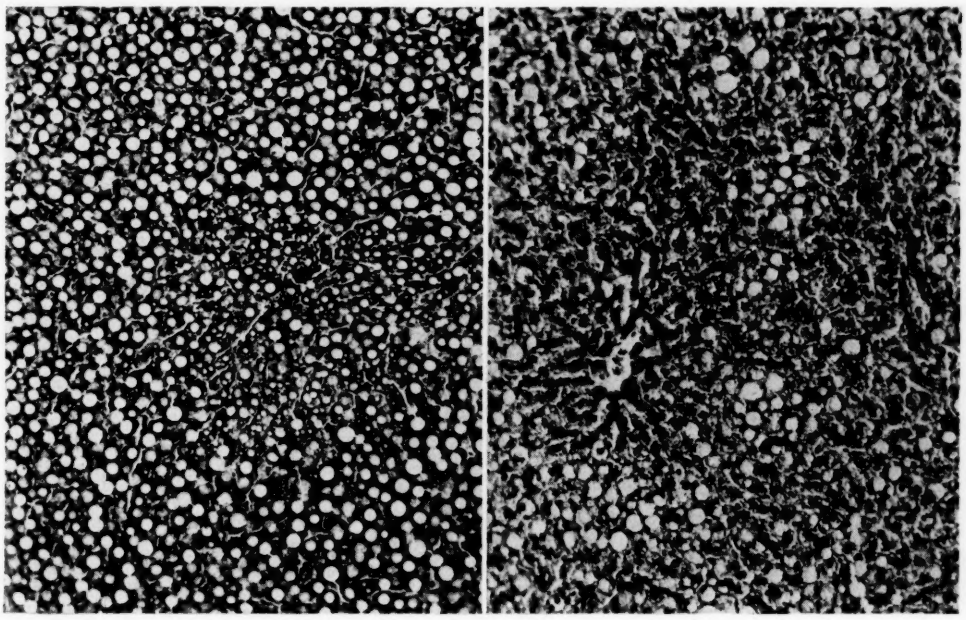
Diets Producing Fatty Liver.—In choline-deficient animals with diffuse fatty metamorphosis of the liver without histologic evidence of liver cell damage (Fig. 4, *A*), the trimethylamine excretion was slightly (but not significantly) reduced. In contrast, the high-fat, low-protein diet given produced diffuse fatty metamorphosis of the liver associated with focal necrosis and variation in staining qualities of the cytoplasm of the nonfatty liver cells (Fig. 4, *B*), and the trimethylamine excretion was significantly reduced. Administration of choline intraperitoneally on the day preceding administration of a test dose of choline reduced the fat content of the liver slightly, but choline orally reduced fat significantly. However, in either case the trimethylamine excretion was not significantly higher than in animals which had not received choline prior to the test dose. Similarly, continuous supplementation of the diet with approximately 5 to 7 mg. of choline per day, an adequate supply, did not prevent fatty metamorphosis, and the trimethylamine excretion was not altered in comparison to rats on the unsupplemented diet. If bromobenzene was added to the high-fat, low-protein diet, fat accumulation was almost completely prevented, but very severe liver damage was noted. The trimethylamine excretion was the lowest of the entire group of chronic liver injury.

DISCUSSION

In rats, 40 per cent of fed choline, regardless of the dose given, is transformed to trimethylamine having no lipotropic activity.[13] Quantitatively, the efficiency of transformation is smaller than in the normal human being who

excretes 60 per cent of fed choline in the urine as trimethylamine. The evidence presented for the human being[2] that this transformation results from the action of intestinal bacteria seems to apply as well to the rat, because parenteral administration of choline leads to urinary excretion of choline, but not to an increase in the basal urinary excretion of trimethylamine. The urinary excretion of parenteral choline is quantitatively comparable to the human being, not exceeding 10 per cent of the dose given.

The transformation of choline to trimethylamine, as accomplished by intestinal bacteria, starts high in the intestinal tract. The content of only a small part of the proximal intestinal tract (just following the entrance of the common duct into the duodenum) is free of bacteria capable of transforming choline to trimethylamine.



A. *B.*

Fig. 4.—Hematoxylin-eosin-stained paraffin sections of rat livers. (×80.) *A*, Choline deficiency. Diffuse fatty metamorphosis with formation of large fat globules. *B*, High-fat, low-protein diet. Almost diffuse fatty metamorphosis of the liver cells, which show variation in staining qualities and focal necroses.

The question arises whether or not the amount of choline not accounted for as trimethylamine in the urine has been absorbed in the intestine. Except in diarrhea, no choline is found in the feces, even if urinary trimethylamine excretion is markedly reduced. Choline added to culture media of intestinal bacteria is completely accounted for after incubation as trimethylamine or choline itself.[4] This speaks against the possibility that intestinal bacteria transform choline to any other compound but trimethylamine. Together these findings indicate that fed choline not accounted for as urinary trimethylamine has been absorbed. Therefore, absorption of ingested choline appears more efficient in rats than in the human being. The experiences with lecithin suggest that choline bound in phospholipids is also more efficiently absorbed than free choline.

It is interesting that choline fed together with fat is more efficiently absorbed than choline fed alone. The important factor is the fatty acids because the admixture of Tween 60 and choline has the same effect, even if the diarrheic action of Tween (with fecal losses of choline) is avoided.

In human beings with liver disease, the trimethylamine excretion after choline ingestion is significantly lower. Because trimethylamine fed in these conditions is readily excreted in the urine, a specific inability to handle this substance in liver diseases can be excluded.[2] The question was raised, therefore, whether the reduced trimethylamine excretion in liver disease is due to altered bacterial action or to increased absorption of choline. The experiments presented exclude, at least in the rat, alteration of intestinal motility as a significant factor in view of the lack of effect of parasympathomimetic drugs. The rat studies indicate that acute hepatic injury has little effect upon the trimethylamine excretion, whereas chronic hepatic injury, or a variety of types and morphologic appearance, consistently decreases the trimethylamine excretion after choline intake. The reduction parallels the degree of liver cell damage. The possibility that this finding may result from lack of biliary substances, especially the bile acids, can be excluded because common duct ligation does not change the trimethylamine excretion.

It is possible in chronic liver disease that choline deficiency or a disturbance of the hepatic fat metabolism, as evidenced by a fatty liver, may cause increased intestinal choline absorption (reflected in reduced trimethylamine excretion). However, choline-deficient and ethionine-intoxicated rats with acute fatty liver have an almost normal trimethylamine excretion. In the fatty liver produced by a high-fat, low-protein diet, the trimethylamine excretion is significantly reduced, but liver cell damage is present to account for the reduction, and adequate supplementation of this diet with choline neither prevents the fatty liver nor increases the trimethylamine excretion. Finally, oral administration of one large dose of choline to rats which have been on a high-fat, low-protein diet reduces hepatic fat significantly without altering the trimethylamine excretion. Peculiarly enough, the parenteral administration of choline has far less effect on hepatic fat and none on the trimethylamine excretion. This series of experiments indicates, therefore, that choline deficiency or available choline depots do not influence significantly the trimethylamine excretion upon choline intake. The same holds true for the absence or presence of fatty metamorphosis of the liver.

It thus appears that chronic liver damage has a significant effect upon the fate of choline in the intestine. This underscores the role which hepatic injury plays upon the intestinal tract as emphasized recently.[14, 15] It cannot be decided at present whether or not the reduced trimethylamine excretion in chronic hepatic injury reflects altered biochemical activity of the bacteria or differences in bacterial strains. The importance of intestinal flora in liver disease has been demonstrated recently by the beneficial effect of its alteration by antimicrobial agents in clinical[16] and experimental[17] liver injury. If altered bacterial action is the cause of the findings presented, the intestine would absorb a larger amount of choline remaining after the choline transformation by bacteria which starts

high in the intestine. However, increased absorption of choline, leaving less for the intestinal bacteria to act upon, cannot be excluded in liver injury. Experiments are under way to measure directly the efficiency of intestinal absorption of choline under normal circumstances and in hepatic injury.

SUMMARY

The fate of exogenous choline was studied in normal rats and in rats with various forms of experimental hepatic injury. Regardless of the dose given, 40 per cent (expressed on a nitrogen basis) of orally administered choline is excreted in the urine in the form of nonlipotropic trimethylamine. The site of the transformation of choline into trimethylamine is localized to the intestinal tract, as in human experiments, in view of the lack of significant urinary trimethylamine excretion after parenteral administration of choline. The content of the entire intestinal tract, with the exception of a short segment in the upper part of the small intestine, is capable of transforming choline to trimethylamine. This transformation is not significantly altered by doses of atropine or prostigmine which significantly alter vitamin A absorption. The trimethylamine excretion is lower if choline is given either in the form of lecithin or together with fat and Tween 60. Since choline is not excreted in the feces (except in diarrhea), even if there is reduced urinary trimethylamine excretion, choline not accounted for as trimethylamine in the urine can be considered absorbed. In acute hepatic injury produced by carbon tetrachloride or bromobenzene, the urinary trimethylamine excretion after choline administration is not significantly altered. After chronic hepatic injury produced by various methods resulting in a variety of histologic phenonena, the trimethylamine excretion is reduced parallel to the degree of liver damage. This reduction is not the result of disturbed bile secretion because ligation of the common duct is without effect. It is not caused by choline deficiency, and there is no evidence that it is related to fatty metamorphosis of the liver. These findings are taken to indicate that hepatic injury influences the activity of the intestinal tract, either through the intestinal bacteria or through alteration of intestinal absorption, leading to increased absorption of choline.

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BIOCHEMICAL ASPECTS OF NASAL POLYPS

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WHENEVER in the past few years nasal polyps have been available we have carried out work to collect data on their water, electrolyte, and nitrogen contents. Although the histopathology of these growths is somewhat familiar, little information is available about their pathogenesis.

Nasal polyps are pendulous projections of the mucous membrane usually having a narrow pedicle, which may be attached to the middle turbinate, or to the uncinate process of the ethmoid bone, or to the walls of the ethmoidal or maxillary paranasal sinuses. Polyps commonly are covered by a thin layer of pseudostratified columnar ciliated epithelium, which often rests upon a thickened basement membrane. In some cases squamous cell metaplasia is found in areas of the epithelium. The bulk of the polyp structure is made up of loose, edematous fibrous tissue in the subepithelial matrix throughout which are varying numbers of young capillaries, eosinophils, plasma cells, and lymphocytes. Some areas of fibrosis may be found, and glandular retention cysts are rather common.

Several theories have been advanced concerning the probable cause of these mucous membrane growths. The ones generally accepted are that they are the result of either allergy or of chronic inflammation, or possibly a combination of both. Chronic suppuration of the paranasal sinuses and trauma also seem to be other factors. It was in an attempt to discover more about the pathogenesis of polyps that the present work was begun.

PROCEDURES AND METHODS

The polyp specimens from thirty-one patients were collected over a period of about two years. The polyps were removed surgically with nasal wire snares and with the aid of Gruenwald forceps. The tissues immediately were dropped into weighed glass-stoppered weighing bottles and sent at once to the laboratory.

The mass of the polyp tissue obtained from any subject varied from 2 to 30 Gm. The number of constituents examined depended upon the amount of the polyp tissue available from each patient. The determinations were rotated in order to obtain representative data for each constituent.

The polyps from each patient were prepared for analyses as follows: The weighing bottle containing the polyps was again weighed and the wet weight of the polyp obtained by difference. The bottle was then placed in a 100° C. thermostatically controlled oven until constant weight was obtained (forty-eight hours). After the determination of the

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water content, the dried mass was transferred quantitatively to a special apparatus[1-3] and crushed. The powder was then returned to the original weighing bottle, placed again in the 100° C. oven two to three hours to remove any water that might have been absorbed during the crushing process, and then stored in a desiccator over activated aluminum oxide. This pulverized tissue was the material used for all of the analyses. Before each aliquot was removed for weighing the sample, the mixture was stirred thoroughly with a stainless steel spatula.

The following determinations were made on the dried polyp tissue: water, chloride, sodium, potassium, calcium, magnesium and total nitrogen. Chloride was determined by the wet ashing method of Van Slyke[4] with the Wilson and Ball modification.[5] Sodium and potassium were determined using a Perkin and Elmer Flame photometer. Calcium determinations were made by the method of Kramer and Tisdall[6]; magnesium by a modified method of Denis[7] and total nitrogen by the micro-Kjeldahl method of Campbell and Hanna.[8] The exact procedures of applying these methods to dried tissue have been given in detail in a previous paper.[9] The approximate amounts of the dry polyp tissue used for the different determinations were as follows: for chloride, 50 mg., for sodium and potassium 100 mg., for calcium and magnesium 200 mg., and for total nitrogen 30 mg.

RESULTS AND DISCUSSION

The analytical data for each of the polyps from thirty-one patients are given in Tables I and II, together with the means with standard deviations. In Table I the values are given in units per kilogram of wet polyp tissue; in Table II in units per 100 Gm. of dry polyp solids. The reason for expressing the data in two ways is for the purpose of comparing the analytical data with blood serum data, antrum cyst fluid data, and other soft tissue data.

In a previous paper[10] we have showed that fluids from the antrum cysts contain approximately the same concentration of both electrolytes and protein as blood serum. Our results bring out the fact that the concentration of the electrolytes in these polyp tissues bear this same relationship to blood serum. Histologically, polyps have both a fluid phase and a cell phase. Considering these tissues, therefore, as soft tissues and comparing their constituents with the constituents of other soft tissues of the body, the nasal polyp chloride and sodium occur in high concentration while the potassium is low; in fact the salt concentrations in these polyps are those characteristic of body fluids and not of soft tissues. These high concentrations of sodium and chloride suggest three possibilities: (1) that all of the chloride is in a space resembling blood in its salt content; (2) that another phase is present which contains both sodium and chloride in ultrafiltrate proportions (connective tissue), and (3) that the sodium and chloride are distributed freely throughout the tissue. The lower concentration of sodium in the polyp tissue and the higher concentration of potassium when compared with the serum suggests that sodium is not distributed freely throuhgout the entire tissue and that the potassium is concentrated in a small portion of it (cells).

If all of the sodium and chloride of the nasal polyp tissue exists in the same proportion as has been found in the ultrafiltrate of serum, the ratio of sodium to chloride in the polyp water should approximate the value of 1.26, which is the serum ultrafiltrate ratio. The chloride of each polyp specimen, expressed in milliequivalents per kilogram of polyp water, was plotted against the sodium of the same specimen, expressed in milliequivalents per kilogram of

polyp water with the pattern developing as shown in the figure. The solid line on the chart has been drawn to represent the respective values of chloride and sodium in an ultrafiltrate of serum. Inspection of the curve reveals that the sodium and chloride values of the majority of the polyps fall remarkably close to this line, which indicates that sodium and chloride exist mainly in the same proportion in polyps as in serum.

On the basis of comparing one kilogram of polyp tissue with one kilogram of any other soft tissue of the body, the potassium and magnesium concentrations are rather low, and indicate a small cellular phase in these tissue masses.

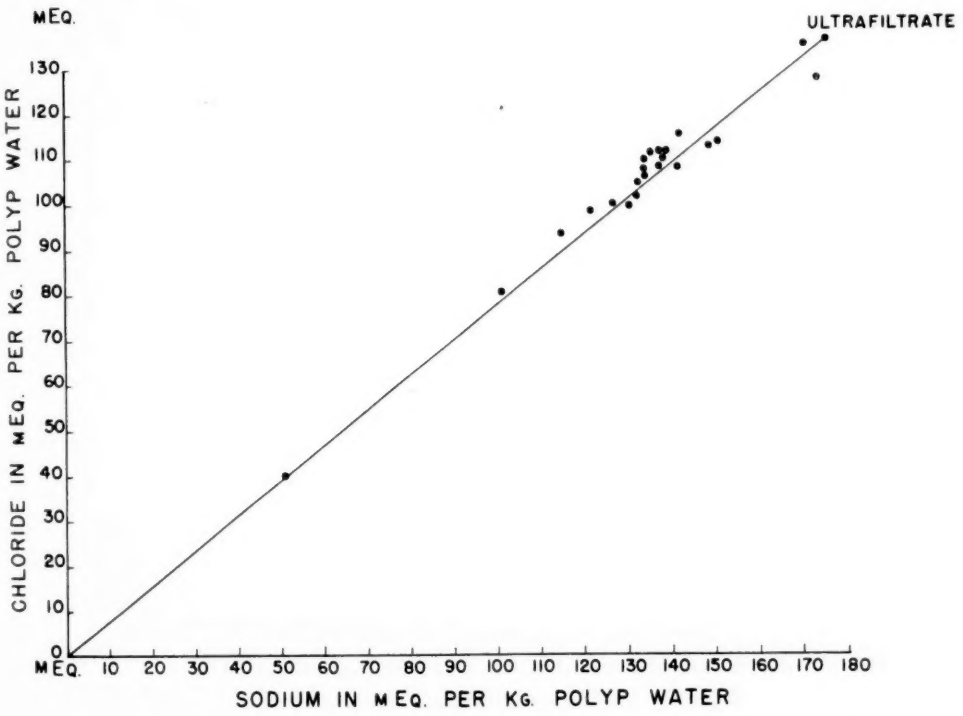

Fig. 1.—The chloride of polyp expressed in milliequivalents per kilogram of polyp water is plotted against the sodium of polyp expressed in milliequivalents per kilogram of polyp water in all patients. The values were calculated from Table I.

The protein content further indicates a low cell mass. Biochemically, the presence of a small cell phase accounts mainly for the differences between nasal polyps and antrum cyst fluids. Although we cannot tell quantitatively at present the cell volume in each kilogram of polyp specimen, the cells are the same in chemical composition for each tissue. This is indicated in Table II. The cellular constituents, nitrogen, potassium, and magnesium, when expressed per 100 grams of dry solids, are very constant with small deviations.

Some of the fluid from nasal polyp tissue was collected and the total nitrogen determined on three different specimens. An average value of 9.0 Gm. of total nitrogen per liter of fluid was found. If all of the fluids from the other polyp tissues were assumed to have this approximate value of nitrogen,

then the remaining nitrogen of the total amount determined would represent the protein solids of the cells. It will be noted that the 9.0 Gm. of total nitrogen found per liter of polyp fluid is not different from the average 9.5 Gm. of protein nitrogen found in the antrum cyst fluids. The finding in the cyst fluids led us to the opinion that the semblance to inflammatory exudates must have been the result of increased permeability of the cyst membranes resulting from infection, bacterial products, or proliferative vascular reactions. The same environmental conditions prevail here. That the presence of infection has caused some of these disturbances has been indicated by the data of Andersen and Bing,[11] who partitioned the total protein of nasal polyps into the albumin and globulin fractions, and found that in some cases the globulin fraction was higher than that in the serum.

From the biochemical point of view, therefore, the fluids of the nasal polyp masses are similar to those of the antrum cyst fluids in electrolyte (Cl, Na, K, Ca) and nitrogen content. The sodium : chloride ratio in both the cyst fluids

TABLE I. ORIGINAL DATA: ANALYSES OF NASAL POLYPS FROM PATIENTS

PATIENT	WATER (GM.)	SOLIDS (GM.)	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	CA (MEQ.)	MG. (MEQ.)	TOTAL N (GM.)	NPN (GM.)
1P	881.8	118.2	102.3	125.0	16.5				
2L	869.3	130.7	99.5	130.3	23.8			16.8	
3S	877.0	123.0	119.7	149.6	20.2				1.4
4J	875.5	124.5	94.7	107.7	26.6				
5C	897.5	102.5		118.0	20.9				
6S	900.0	100.0	84.8	103.0	15.6				
7B	866.8	133.2	93.2	114.9	33.3			17.6	1.2
8V	874.0	126.0	86.2	105.6	27.9	5.56		16.3	1.1
9S	878.7	121.3	112.5	151.4	18.2			16.7	
10T	861.0	139.0	97.5	127.5	25.9			19.0	
11L	876.0	124.0	90.2	110.9	21.0			16.3	
12D	885.2	114.8	96.8					14.5	
13R	856.0	144.0	91.5					19.3	
14G	878.7	121.3				4.84	6.62	16.1	
15C	890.8	109.2				4.48	6.53		
16H	878.9	121.1	99.5			4.63	12.83	16.1	
17H	904.4	95.6	95.3					12.1	
18H	891.7	108.3	105.7					14.0	
19S	893.4	106.6		115.8	20.5				
20R	912.0	88.0	102.1	124.5	12.1				
21S	891.5	108.5	94.2	117.5	17.4				
22M	878.5	121.5	87.7	114.8	26.0				
23E	897.3	102.7	95.5	119.5	17.9				
24K	865.9	134.1	99.2	119.0	21.5			19.2	
25H	901.8	98.2	100.7	124.5	14.9				
26B	888.9	111.1	99.4	119.6	21.5				
27P	890.9	109.1	88.5		22.4			13.8	
28D	889.4	110.6				6.41	5.00		
29M	898.8	101.2							
30D	899.8	100.2	98.4	123.5	14.1	5.78	4.38		
31W	899.0	101.0	97.3	126.8	21.7				
Mean*	885.5	114.5	97.5	121.4	20.8	5.28	7.07	16.2	
	13.5	13.4	7.6	12.0	4.9	0.69	3.00	2.1	
Antrum Cyst Fluid[10]	940.8	59.2	100.5	141.8	4.54	4.54	1.80	9.65	
Serum[10]	937.0	63.0	100.5	135.0	4.55	5.40	1.60	10.6	

Values are expressed in units per kilo of polyps.
*Standard deviation.

TABLE II. ANALYSES OF NASAL POLYPS

PATIENT	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	CA (MEQ.)	MG. (MEQ.)	TOTAL N (GM.)
1P	86.7	105.6	14.0			
2L	76.2	99.6	18.2			12.8
3S	97.3	121.6	16.4			
4J	76.1	86.5	21.3			
5C		115.1	20.4			
6S	84.8	103.0	15.6			
7B	69.6	86.2	25.0			13.2
8V	68.4	83.8	22.1	4.41		12.9
9S	92.8	124.8	15.1			13.5
10T	70.2	91.8	18.6			13.7
11L	72.8	89.6	17.0			13.2
12D	84.4					12.6
13R	63.5					13.4
14G				3.99	5.46	13.2
15C				4.10	5.98	
16H	82.2			3.82	10.60	13.3
17H	99.9					12.6
18H	97.6					12.9
19S		108.6	19.2			
20B	116.0	141.4	13.7			
21S	86.8	108.3	16.0			
22M	72.2	94.6	21.4			
23E	96.7	116.3	17.5			
24K	71.2	88.8	16.0			14.3
25H	102.5	126.7	15.2			
26B	89.4	107.7	19.4			
27P	81.1		20.5			12.6
28D				5.80	4.52	
30D	98.3	123.4	14.0	5.77	4.37	
31W	96.4	125.5	21.5			
Mean*	86.2	107.1	18.1	4.71	6.19	13.1
	13.0	15.9	3.0	0.83	2.3	0.5

Values are expressed in units per 100 grams of dry solids.
*Standard deviation.

and the nasal polyps is the same as that in blood, indicating the presence of a phase in both the cyst fluid and nasal polyps similar in composition to blood. This suggests the interesting possibility that the same pathological conditions foster the formation of both of these phenomena.

SUMMARY

Nasal polyps have been analyzed for water, chloride, sodium, potassium, calcium, magnesium, and total nitrogen content. The sodium : chloride ratio in the polyps is approximately the same as that in blood, indicating the presence of a phase similar in composition to that of blood.

From a biochemical point of view, the fluids from nasal polyp masses are similar in electrolyte and nitrogen contents to those of the antrum cyst fluids.

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MODIFIED GLOBIN

II. Chemical Changes in Human Globin Produced by Alkaline Modification

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INTRODUCTION

IN AN earlier paper[1] of this series the method of preparation of a modified globin from human erythrocytes has been described. In this paper we present evidence upon the nature of the chemical changes that occur when globin hydrochloride is treated with alkali.

The major change that occurs when acid globin is treated with alkali, under controlled conditions, is one of partial deamidation of the protein. Accompanying this there is a partial racemization of some of the amino acids. There is some cleavage of the molecule to units with a molecular weight of 10,000 to 14,000, while the major part of the protein is reduced to a molecular weight of approximately 34,000. With the deamidation of the protein, acidic groups are liberated, and the pH at which the protein begins to precipitate is reduced to a range of 6.5 to 5.5.

MATERIALS

The elementary composition of the acid globin used in preparing modified globin is quite constant. It contains no phosphorus, and only traces of iron, presumably due to minor quantities of hemin chloride not extracted during its preparation. Table I describes the elementary composition of two representative samples of acid globin, and one lot of modified globin.

Table I. Elementary Composition of Globin Hydrochloride and Modified Globin

Analysis	Acid globin no. 203 (per cent)	Acid globin no. 242 (per cent)	Modified globin no. 242 (per cent)
Total nitrogen, Kjeldahl (free of moisture, ash, cation or anion)	17.10	17.06	16.59
Total sulfur, Pregl (free of moisture, ash, cation or anion)	0.66	0.64	0.66
Chlorine	5.37	5.34	1.12
Ash	0.18	0.41	4.72
NaCl	——	——	1.83
Na-ion	——	——	1.11

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This paper is the twenty-fourth of a series on blood, plasma, and plasma substitutes from the Laboratory of Clinical Pathology and the John S. Sharpe Research Foundation of the Bryn Mawr Hospital. The work described here was aided by a grant from the John S. Sharpe Research Foundation and was in part carried out under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Bryn Mawr Hospital and a Contract with the Army Medical Research and Development Board.

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The value for total nitrogen of acid globin is higher than that reported by Beach and associates,[2] namely 16.58 per cent, which value had been corrected for anions but not for moisture. The sulfur content is in agreement with the values reported by Beach and co-workers[2] and Roche[3] for human globin. In the modified globin there has been a significant decrease in nitrogen content, with no change in sulfur content. The high ash content is due to incomplete dialysis of the finished preparation of modified globin prior to drying for analysis.

METHODS, RESULTS, AND DISCUSSION

The rate of chemical modification of acid globin had been studied under conditions in which only one factor was changed at a time. Variations in protein concentration, hydroxyl ion concentration, and temperature, all materially alter the rate of chemical change. Table II indicates the extent of the change in the precipitability characteristics of globin occurring with changes in the time of treatment.

In the standard method of preparation modification is continued for a period approximating twenty-seven hours. The data in Table II show that, at this time, the processes of chemical changes in the protein are by no means complete.

TABLE II. RATE OF MODIFICATION OF ACID GLOBIN WITH ALKALI EXPERIMENTAL CONDITIONS: TOTAL N = 0.45 M/L; TOTAL NAOH = 0.26 M/L; 37° C.

TIME (HRS.)	ACID† CLOUD POINT (PH)	PER CENT OF TOTAL N NOT PRECIPITATED BY *			
		H_2WO_4	TRICHLOR-ACETIC 9 PER CENT	TRICHLOR-ACETIC 2.5 PER CENT	HPO_3 0.15N
1	9.0	0.6	0.9	0.9	0.9
20	6.9	3.5	8.2	16.3	12.3
27	6.6	5.0	10.8	19.5	14.8
41	6.2	6.5	15.4	24.6	20.3
72	5.6	8.2	19.5	39.0	27.5
120	5.5	10.9	26.2	41.2	35.6
168	5.5	12.7	29.5	- - -	40.6
7 wks.	5.5	31.9	31.9	63.2	- - -

*The protein solutions were adjusted to pH 6.8 to 7.2 prior to analysis. All precipitations conducted at a 1:10 dilution. The final concentrations of TCA were either 9 or 2.5 per cent.
†The pH at which the first visible turbidity occurred when the solution was titrated.

When other types of proteins are treated with alkali, comparable in concentration to that used here, certain amino acids are known to be decomposed. The hydroxy amino acids, serine and threonine are susceptible to destruction by alkali,[4] and cystine in many proteins is destroyed by alkali treatment. Accordingly the nitrogen distribution, and the content of certain amino acids was determined in a representative sample of globin hydrochloride and modified globin. The methods of analysis used were chosen to show any differences in the two preparations, rather than to produce analytical values of high accuracy for specific amino acids. The data obtained are summarized in Table III.

The protein was hydrolyzed with ten parts of 5M HCl in the presence of 0.4 parts of Norite at 125° C., for sixteen hours in a sealed tube. Longer periods of heating did not result in any further increase in amino nitrogen. The hydrolysates were also suitable for the optical rotations to be reported below.

The amide N was determined by distillation, in vacuo, from an alkaline solution [$Ca(OH)_2$] after previous removal of the excess HCl. The quantity found for the acid globin is in excellent agreement with that reported by Hunter and Borsook.[5] From the figure for modified globin it can be seen that some of the amide N has been removed by the treatment with alkali. More details upon the rate of this process will be presented below.

The diamino acids were determined by phosphotungstate precipitation by Van Slyke's[6] method, correcting the values for solubility of the phosphotungstates.[7] There was no variation in the diamino-N found in the two preparations. The value agrees with that found by Hunter and Borsook.[5] Lysine-N was estimated[6] as two times the difference between the thirty-minute amino N and the α-amino N (ninhydrin) of the diamino N fraction; nitrite containing KI was used according to Kendrick and Hanke[8] to avoid interference from any cystine present. Alkali treatment did not produce any changes in the lysine content of modified globin. The value agrees with those reported by others.[2, 5]

The nitrogen of proline and oxyproline was estimated as the difference between the total N and the amino N of the monoamino acid fraction, while aspartic acid N was considered to be the difference between the apparent α-amino N (ninhydrin) and the total N of this fraction. Tyrosine was estimated by the method of Folin and Marenzi.[9] The treatment with alkali has not caused any destruction in these amino acids.

The hydroxyamino acids, serine and threonine, were estimated by the method of Nicolet and Shinn.[10, 11] There was a definite decrease in the serine content of modified globin, while the change in threonine content approaches the limit of precision of the method. These variations in hydroxyamino acid content are less than those observed when silk was treated with alkali.[12]

The alkali-lability of the cystine, in many proteins, is a well-established fact. In the modified globin no evidence of cystine destruction was observed. The total sulfur and the cystine and methionine-S distribution was identical within the limits of the analytical procedure.[13]* No lanthionine was formed as

TABLE III. NITROGEN DISTRIBUTION AND DEGREE OF RACEMIZATION OF FRACTIONS OF ACID GLOBIN AND MODIFIED GLOBIN

ANALYSIS	ACID GLOBIN NO. 242 (% OF TOTAL N)	MODIFIED GLOBIN NO. 242 (% OF TOTAL N)
"Amide" N	5.34	3.81
Di-amino acid N	31.5	31.5
Mono-amino acid N	59.6	61.3
Lysine N	10.1	10.3
Proline and oxyproline N	2.3	2.3
Aspartic acid N	8.8	8.5
Tyrosine N	1.58	1.59
Threonine N	4.12	4.00
Serine N	3.33	2.85
Cystine N	0.16	0.17
Methionine N	0.12	0.12
Sulfate sulfur	0.00	0.00
DEGREE OF RACEMIZATION	PER CENT	PER CENT
Total hydrolysate	0.0	34.3
Mono-amino acids	0.0	37.3
Diamino acids	0.0	35.1

*We wish to express our appreciation to Dr. Erwin Brand for determining the sulfur distribution in these preparations.

is shown by the fact that the sulfur of cystine and methionine account for the total sulfur found.

Levene and Bass[14] have shown that different proteins vary in their degree and rate of racemization under the influence of alkali. Their procedure was employed to determine the degree of racemization that has occurred in modified globin. In addition, hydrolysates of acid globin, the regularly prepared modified globin and a globin solution that had been treated for fifteen days with 5M sodium hydroxide, were separated into the mono-amino and diamino-acid fractions by Van Slyke's procedure. The data are presented in Table III. The optical rotation of the total hydrolysate of modified globin showed it to be 34.3 per cent racemized. When the amino acid mixture was fractionated it is apparent that the mono-amino acids have undergone a greater change, 37.1 per cent, than have the diamino acids. The mechanism of racemization proposed by Levene[14] suggested that amino acids located toward the end of peptide chains will be less racemized, under the influence of alkali, than ones located in the middle of the chains.

TABLE IV. FREE AMINO N, TOTAL ACID AND BASIC GROUPS, AMINO AND AMIDE N, OF HYDROLYSATES, AND DEGREE OF RACEMIZATION OF ACID GLOBIN AND MODIFIED GLOBIN

PREPARATION		"FREE AMINO" N PER CENT OF TOTAL N	DYE-COMBINING CAPACITY MEQ. PER GM. PROTEIN N		AMINO N OF HYDROLYSATE PER CENT OF TOTAL N	AMIDE N OF HYDROLYSATE PER CENT OF TOTAL N	RACEMIZATION (PER CENT)
			ACID GROUPS	BASIC GROUPS			
Acid globin	242	5.10	18.4	9.95	81.7	5.34	0.0
Globin	242	4.95	22.9	9.15	82.2	3.81	34.3
Globin	854	5.32	25.2	9.00	82.0	3.71	36.6
Globin	954	5.14	19.6	8.73	81.6	3.96	25.7
Globin	1154	5.17	24.1	9.02	80.7	3.82	28.7
Globin	1254	5.05	21.2	9.17	80.7	3.99	32.2
Globin*	218C	5.70	19.2	7.87	76.3	2.67	63.0

*Sample treated with alkali for three times the normal period, i.e., seventy-two hours.

The degree of racemization of several preparations of modified globin is reported in the last column of Table IV. A value approaching 30 per cent can be expected in preparations produced by the standard procedures. When the period of alkali treatment was increased threefold racemization had increased to 63 per cent (Globin 218C).

The free amino nitrogen of modified globin showed little or no increase in products produced under the standard-time treatment. With longer treatment, as in lot 218C, a considerable increase was observed. This indicated that the major component in the purified globin solutions was not hydrolyzed extensively unless alkali treatment was continued for longer periods.

One of the desired changes in acid globin under alkali treatment was the lowering of the isoelectric point and the resultant increase in solubility at pH 7.0. This could be accompanied by a decrease in total free basic groups, an increase in total free acidic groups, or both could be changed. The method of Fraenkel-Conrat and Cooper[15] was employed to determine the change in number

of total acidic and basic groups. This procedure determines the capacity of a protein to combine with basic or acidic dyes. The dye-binding method gave values agreeing within ±5 per cent. The values for the globins, Table IV, show a rather wide scattering, especially in the figures for total acidic groups. However, a definite increase in the total acid groups, and a slight decrease in total basic groups, is evident. Globin, lot 218C, showed a marked decrease in basic groups, perhaps indicating selective hydrolysis of basic amino acids from the ends of peptide chains.

Perlmann[16] has shown that the amount of meta-phosphoric acid combining with protein, to produce an insoluble complex, can be used as a measure of the basic groups in a protein. Six preparations of acid globin bound 134.8 moles HPO_3 per 10^5 grams of protein, with a range of 130.0 to 138.0. Five different

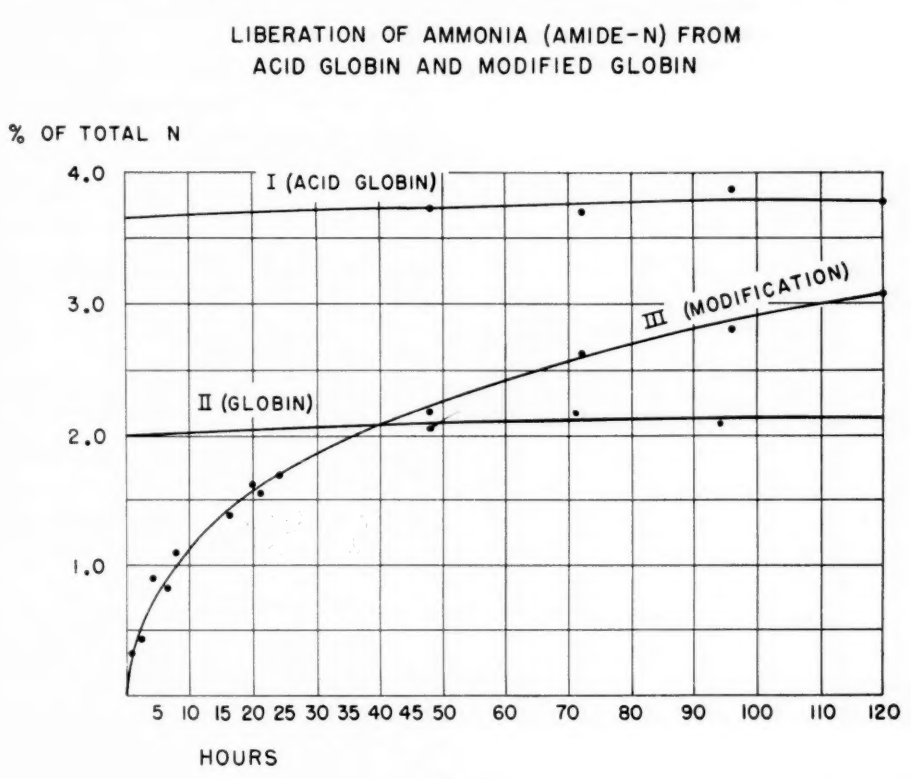

Fig. 1.

lots of modified globin bound 134.5 moles HPO_3, range 131.9 to 136.9. Cohn, Salter, and Ferry[17] have determined the acid-combining groups in globin, by other means, and found 138×10^{-5} per gram of protein.

The amino N of the hydrolysates was determined, but no significant change was observed on any of the globins treated for the usual length of time. The overtreated sample, 218C, showed a definite decrease in percentage of amino N.

The values for the amide-nitrogen of the hydrolysates of various modified globins (Table IV) are quite consistent, and reflect the loss of ammonia from the amide linkages of acid globin. The percentage of ammonia removed by

alkali treatment and purification corresponds closely to the figure obtained by a different method reported below. Further loss of amide-N occurred when alkaline treatment was continued for a longer period of time (218C).

In a further study of the liberation of amide N during alkaline modification of acid globin the method of Warner and Cannan[18] was employed. They observed that ammonia was liberated at a rapid rate early in the period of alkaline treatment, and finally was evolved at a slow and constant rate over a period of several days. The early phase was ascribed to the hydrolysis of amide linkages, while the later and slower rate was caused by the decomposition of certain amino acids, e.g., hydroxy and sulfur-containing amino acids. Extrapolation of the straight part of the curve to zero time would give the actual value for the true amide nitrogen. To remove ammonia at stated intervals the alkaline solution was aerated for 1 hour, at the rate of 100 liters of air per hour, using n-decanol as an antifoam agent. The solutions contained 5 per cent of protein in 1M NaOH and were incubated at 37° C. Preliminary experiments indicated that the liberation of ammonia occurred at a constant rate after two days.

The results of such an experiment upon an acid globin and a modified globin are shown in Curves I and II of Fig. 1. Extrapolation to zero time gives a value for true *amide-N* for acid globin of 3.68 per cent of the total protein N and 2.00 per cent for the corresponding modified globin. The difference, 1.7 per cent, can be ascribed to the ammonia liberated during the standard alkaline treatment. To demonstrate this directly the same acid globin was treated with alkali under the actual conditions of modification, i.e., 5 per cent in 0.15M "free" NaOH, or 0.26M NaOH total alkali, and the ammonia liberated at the stated times was determined. The results are plotted in Curve III of Fig. 1. After twenty-four hours of alkali treatment, 1.7 per cent of the total nitrogen was liberated as ammonia, or only 45 per cent of the available ammonia from the total amide linkages. Even after five days only 85 per cent of the amide groups were hydrolyzed under these same conditions of alkali treatment. There is excellent agreement between the twenty-four hour figure in Curve III with the differences between Curves I and II.

The free acidic groups in the protein increase with the liberation of ammonia from the amide groups. This has been demonstrated in Table IV, by the results of dye-binding measurements. There was no strict equivalence between the two values, the amide ammonia accounting for only about one-half of the increase in free acidic groups. No explanation for this apparent discrepancy can be given, though it may be pointed out that the values for the free acidic groups show a rather wide variation, and the limits of error of the dye-binding method are certainly larger than those in the determination of the ammonia liberated and determined by three independent methods.

The data for free amino nitrogen in the preparations listed in Table IV indicate that a seventy-two hour treatment with alkali significantly increases this value. A study was therefore made of the rate at which peptide bonds are liberated under the standard method of alkali treatment of acid globin, i.e., 5 per cent protein in a solution containing 0.15M free-NaOH incubated at 37° C. Samples were withdrawn at twenty-four hour intervals, and the free amino

nitrogen was estimated by Van Slyke's nitrous acid method after prior removal of the amide nitrogen. A sample of the same protein was hydrolyzed by acid and the total amino nitrogen was determined. The increase in the percentage of free amino nitrogen, expressed as per cent of the total amino groups bound in peptide linkage, was 0.56, 1.02, 1.76, 2.23 and 2.84 for each twenty-four hour interval up to one hundred twenty hours. Only about 0.5 per cent of the amino groups was liberated in the normal period of treatment. Globin seems to be remarkably slowly hydrolyzed when compared to the rates of hydrolysis obtained on a number of proteins under similar conditions by Levene and Bass.[14]

In view of the fact that a rather wide variation in degree of racemization was observed in the different samples of globin, listed in Table IV, a more careful study was made of the rate at which racemization progressed. The acid globin was modified as usual, neutralized, frozen, and dried from the frozen state. The dried protein was hydrolyzed as described above and the optical rotation determined. The rotation obtained after the fifteen-day alkaline treatment described earlier was considered to equal 100 per cent change. The percentage racemization at the various times was: twenty hour, 34.3; twenty-

ELECTROPHORETIC PATTERN-MODIFIED HUMAN GLOBIN
3
S
4
1 2
LOT NO. 87645 - VERONAL BUFFER pH 8.5, IONIC STRENGTH 0.1, POTENTIAL GRADIENT 10.39 V/CM. DESCENDING BOUNDARY. COMPONENT NO. 1, 3.7 %
μ = 2.17 CM²/V/SEC. COMPONENT NO. 2, 4.3 %
μ = 2.04 CM²/V/SEC. COMPONENT NO. 3, 86.7 %
μ = 4.88 CM²/V/SEC. COMPONENT NO. 4, 5.3 %
μ = 6.96 CM²/V/SEC. S = SALT BOUNDARY

Fig. 2.

four hour, 39.7; forty-eight hour, 53.4; seventy-two hours, 63.9; ninety-six hours, 69.0; one hundred and twenty hours, 77.6; and two hundred and eighty-eight hours, 98. Thus it is apparent that at the period of modification normally employed, i.e., twenty-four to twenty-seven hours, racemization is still progressing rapidly. Small variations in the time of treatment, and also in the actual concentration of alkali used, can account for the variations in the degree of racemization observed in the samples listed in Table IV.

Hemoglobin has a molecular weight of 68,000. During the process of alkaline modification of globin, which appeared to be predominantly a process of deamidization, there was an associated cleavage of the molecule. A small amount of the protein was cleaved to units of relatively low molecular weight. This fraction can be removed largely by dialysis through Visking membranes. They

also can be removed from the major component by salt fractionation. The molecular weight of 80 per cent and more of alkali-treated globin, as determined by osmotic, ultra centrifugal, and viscosity studies, is around 34,000, or about half that of hemoglobin.[19-21] Electrophoretic patterns have been determined[22] in phosphate and veronal buffers, ionic strength 0.1 or 0.2,, at various potential gradients. In every pattern there was a major component, accounting for 82 to 92 per cent of the globin, with a mobility of approximately 4.6×10^{-5} cm./volt/sec. In phosphate buffer two other minor components were found regularly with mobilities of about 1.7 and 2.5×10^{-5} cm./volt/sec., while with veronal buffers a fourth component could be distinguished of a mobility of about 6.9×10^{-5} cm./volt/sec. Fig. 2 illustrates a representative electrophoretic pattern for modified human globin determined in veronal buffer.

SUMMARY

Among the changes that occurred when acid globin was modified by treatment with alkali was a cleavage of the molecule to units of approximately 34,000 in molecular weight. Smaller molecules could be removed by dialysis or by salt fractionation.

The chemical changes that occurred in the major component of the protein were associated predominantly with a deamidation with a liberation of ammonia. Only a few peptide bonds were hydrolyzed—about 0.5 per cent of total amino nitrogen became free. There was a partial racemization of the mixed amino acids; but there was little evidence of destruction of particular amino acids. There was some decrease in the hydroxy amino acids, serine and threonine, but no change in the sulfur amino acids was observed.

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INFLUENCE OF CEREBROSPINAL FLUID UPON CEREBRAL BLOOD-FLOW DETERMINATION

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IN THE course of a large number of internal jugular vein punctures,[1] performed for the determination of cerebral blood flow by the nitrous oxide method,[2, 3] the intracranial subarachnoid space was inadvertently entered through the jugular foramen on several occasions. These incidents brought to our attention the feasibility of deliberately obtaining intracranial cerebrospinal fluid through this approach in order to investigate its possible importance as a source of error in the nitrous oxide method of cerebral blood flow estimation.

METHOD

The jugular foramen can be entered and cerebrospinal fluid obtained by one of two approaches. In the first one, a short-bevelled 19 gauge 2½ inch needle is inserted 10 to 12 mm. below the anterior edge of the tip of the mastoid process at an angle of 40 to 45 degrees to the sagittal plane, and in a lateral plane through the posterior edge of the external auditory meatus. Penetration of the wall of the superior bulb of the internal jugular vein may be confirmed by aspiration of venous blood. Further insertion of the needle to a depth of 2¼ inches or more without encountering bone usually indicates that the jugular foramen has been successfully traversed. In the second approach, a 3-inch needle is inserted just lateral to the angle of the jaw, at an angle of 40 to 45 degrees to the sagittal plane and parallel to the descending ramus of the mandible, and passed upward and posteriorly, traversing as above the internal jugular vein before entering the jugular foramen medial and posterior to the styloid process. If the needle has passed through the foramen, cerebrospinal fluid may be aspirated. The fluid thus obtained is assumed to be cisternal fluid.

In five of this series, after a blank sample of cerebrospinal fluid was taken, the needle was partly withdrawn until the tip, which in all cases but one had traversed the superior bulb of the internal jugular vein, was within the lumen of that vessel so that a blank cerebral venous blood sample could be obtained. After the subject had inhaled for ten minutes a standard gas mixture containing 15 per cent nitrous oxide, 21 per cent oxygen, and 64 per cent nitrogen, an "equilibrated" cerebral venous blood sample was taken through the jugular needle which was immediately reinserted through the jugular foramen so that another 2.5 ml. cerebrospinal fluid specimen could be obtained. In cases 4, 8, and 9, the needle tip was permitted to remain in the intracranial subarachnoid space and a separate internal jugular vein puncture was performed on the opposite side. In all but cases 4 and 7 a blood sample was also taken at this time from a needle which had been placed in the femoral artery, and in cases 5 and 6 cerebrospinal fluid was obtained from a needle previously placed in the lumbar sac. In case 7, only lumbar cerebrospinal fluid and cerebral venous blood were compared, and samples were withdrawn after ten minutes of inhalation of the gas mixture. In case 4, four consecutive, continuously drawn specimens of intracranial subarachnoid spinal fluid were obtained during the simultaneous removal of four continuous samples of internal jugular venous blood from the opposite side. In cases 8 and 9 a needle

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was also placed in a lateral cerebral ventricle through trephine openings made, respectively, three and two months previously. Intraventricular and intracranial subarachnoid fluid and internal jugular and arterial blood were sampled at 2.5, 5, 7.5, and 10 minutes after administration of the gas mixture was begun. In five of the nine subjects cerebral blood flow was determined during these experiments; in two others it had been determined shortly before the cerebrospinal fluid investigation.

The method of Kety and Schmidt,[2] as modified by Scheinberg and Stead,[3] for determination of cerebral blood flow was employed. Nitrous oxide content of the various samples was measured manometrically by Kety's[4] modification of the method of Orcutt and Waters.[5]

RESULTS

Of ten cases intended for this series in which jugular foramen puncture was essayed, four attempts were immediately successful, two required a single redirection of the needle, and two were successful only after two or three redirections. Failure was encountered in two subjects. Not counted were two cases in which attempts were abandoned after two redirections of the needle, because the subjects experienced discomfort. In all subjects in whom the foramen had once been entered and the needle then partly withdrawn for blood sampling, re-entry was immediately successful. In no instance were any important sequelae noted; two subjects experienced short paroxysms of coughing, and another exhibited transient (forty-five seconds) bradycardia and hypotension.

TABLE I. BLOOD AND CEREBROSPINAL FLUID NITROUS OXIDE CONTENT AFTER TEN MINUTES OF INHALATION OF 15 PER CENT N_2O

CASE	CBF	ART.	VEIN	CIST.	S_{C10}	VENT.	S_{V10}	LUMB.	S_{L10}
1	36.1	3.06	2.60	2.08	.80				
2	43.5	4.80	4.40	3.12	.73				
3	30.5	4.99	4.30	2.65	.62				
4	41.6		3.78	2.10	.56				
5*	36.1	1.14	0.86	0.57	.66			0.28	.33
6		4.56	4.28	2.36	.55			0.70	.16
7			2.44					0.13	.05
8	20.7	4.49	3.88	2.75	.71	.95	.24		
9	27.7	4.55	3.67	1.36	.37	.06	.02		

CBF = Cerebral blood flow.
Art. = Arterial N_2O, volumes per cent.
Vein = Internal jugular venous N_2O, volumes per cent.
Cist. = Intracranial subarachnoid ("cisternal") cerebrospinal fluid N_2O, volumes per cent.

$$S_{C10} = \frac{\text{Cist.}}{\text{Vein}}$$

Vent. = Intraventricular cerebrospinal fluid N_2O, volumes per cent

$$S_{V10} = \frac{\text{Vent.}}{\text{Vein}}$$

Lumb. = Lumbar cerebrospinal fluid NO, volumes per cent

$$S_{L10} = \frac{\text{Lumb.}}{\text{Vein}}$$

*Gas mixture administered by catheter through tracheotomy tube.

Comparison of arterial and cerebral venous nitrous oxide concentrations, after ten minutes of gas administration, with that of the intracranial subarachnoid cerebrospinal fluid is shown in Table I; in three cases the ten-minute nitrous oxide content of lumbar cerebrospinal fluid is also shown, and in two other cases the ten-minute nitrous oxide concentration of intraventricular fluid is indicated. The mean ratio of cisternal nitrous oxide concentration to cerebral

venous nitrous oxide concentration was 0.625. The standard deviation was 0.125, placing 95 per cent of the cases within ± 0.25 of the mean. This data suggests a correlation between the cerebral venous and cisternal nitrous oxide content. There was no evident correlation of the cisternal nitrous oxide concentration with the cerebral blood flow (which was low in these subjects, four of whom had persistent post-traumatic coma, and five of whom had cerebral vascular disease).

DISCUSSION

The possible hazards of jugular foramen puncture were carefully considered before this investigation was undertaken. The potential risks are as follows: (a) infection; (b) injury to the cranial nerves as they exit through the jugular foramen; (c) bleeding into the subarachnoid space; and (d) direct injury to the brain. In five cases of this series in which the needle was twice inserted through the same jugular foramen within fifteen minutes, there was no evidence of bleeding into the subarachnoid spinal fluid; nor was there any evidence of subsequent intracranial bleeding. The rather poor conformity ebtween the inferior surface of the brain and the floor of the skull makes the last complication unlikely. The only complication resulting from the procedure was that previously referred to of transient vagal irritation seen in three cases. Because of the comparative uncertainty of the technique and its potential dangers, the procedure cannot be generally recommended as a substitute for cisterna magna puncture.

The results of the study employing the above-described techniques clearly indicate that an appreciable cerebrospinal fluid concentration of nitrous oxide, as compared to that in the cerebral venous blood, is attained by the end of 10 minutes of inhalation of the gas in 15 per cent concentration. Thus, during cerebral blood flow determination the blood supply to the brain contributes not inconsiderable quantities of nitrous oxide to the cerebrospinal fluid; this not only influences the denominator of the general equation for the Fick principle,

$$TF = \frac{(Q_B)u}{\int o^u (A\text{-}V)dt}$$

(in its application to cerebral blood flow determination[2] "A-V" is the cerebral-blood arteriovenous nitrous oxide difference), but also modifies the significance of the numerator so that it represents the quantity of nitrous oxide taken up by the entire brain *and intracranial subarachnoid fluid* in time "u" and not by the brain alone. The net effect upon the whole equation is to cause "TF" to represent blood flow related to the total intracranial contents.

Although "$(Q_B)u$" cannot be directly obtained in man, satisfactory information might be obtained from knowledge of the cerebral venous blood nitrous oxide concentration "V_u" at the end of the inhalation period, with consideration of the following factors:

F_1, the fraction of the intracranial contents represented by brain tissue,
F_2, the fraction of intracranial contents represented by cerebrospinal fluid,
S_1, the nitrous oxide partition coefficient between brain and blood at 10 minutes,
S_2, the nitrous oxide ratio between cerebrospinal fluid and blood at 10 minutes.

In terms of 100 gm. units of intracranial contents, where Wt is the total weight of these contents in grams and V_u is expressed as milliliter nitrous oxide per 100 milliliters of blood,

$$\frac{(Q_B)u.100}{Wt} = V_u.S_1.F_1 + V_u.S_2.F_2.$$

The value for blood flow of 100 Gm. of cerebral tissue alone may then be obtained by substituting the above into the general equation for the Fick principle and multiplying by $\frac{1}{F_1}$ the formula then becomes

$$CBF = \frac{100V_u}{\int o^u (A\text{-}V)dt} \times \frac{S_1F_1 + S_2F_2}{F_1}$$

in which cerebral blood flow is expressed as milliliter blood/min./100 Gm. brain.

Although the subjects of this investigation were abnormal, it is difficult to entertain on this basis the objection that normal individuals might not develop at least as high an intracranial cerebrospinal fluid nitrous oxide content as seen in this series. Judging from the data, even with allowance for the markedly delayed appearance of appreciable amounts of nitrous oxide in the intraventricular fluid, the approximate value for S_2 of 0.5 may be generally applicable under normal circumstances; Kety and associates[6] have established for S_1 the value of unity. The accepted range of cerebrospinal fluid volume is 90 to 150 c.c. in normal individuals[7] in whom about 75 to 125 c.c. is within the cranium. With the normal range of brain weight from 1,100 to 1,700 Gm.,[7] and considering the cerebrospinal fluid to have a specific gravity of 1, F_1 is determined to be about 0.93 and F_2 about 0.07. Therefore, substituting in the "corrected partition coefficient" of the formula modified above,

$$\frac{1 \times 0.93 + 0.5 \times 0.07}{0.93} \times 100 = 103.8\%$$

indicating that the normal cerebral blood flow is about 3.8 per cent higher than ordinarily calculated from the data of the nitrous oxide method. Although this is a completely inconsequential error in normal subjects, its basis suggests that the error might be of considerable magnitude in the presence of a pathological increase in cerebrospinal fluid volume, particularly if at the expense of the brain tissue. Thus, in cerebral atrophy F_1 may fall to a value of 0.8 while F_2 rises to 0.2. If the S values were not to change appreciably,

$$\frac{1 \times .8 + .5 \times .2}{.8} \times 100 = 112.5\%$$

so that the true cerebral blood flow in such a case would be about 12.5 per cent higher than the ordinarily calculated value. However, the rather surprising finding that only very small amounts of nitrous oxide appear in the intraventricular fluid suggests that S_1 may be much lower than 0.5 in cerebral atrophy or internal hydrocephalus, in which there occurs marked dilatation of the ventricles. In the hypothetical example previously cited, two-thirds of the

intracranial cerebrospinal fluid might be in the ventricles (S_{v10}, ca. 0.5); S_i would therefore be approximately 0.25 and in such an instance

$$\frac{1 \times 0.8 + 0.25 \times 0.2}{0.8} \times 100 = 106.2\%$$

so that the result would be only 6.2 per cent higher than the ordinarily calculated value, not 12.5 per cent. Nevertheless, the larger error might well be present in external hydrocephalus.

It should be emphasized that the results of these experiments indicate only a potential source of error in cerebral blood flow determination in the presence of increased quantities of extraventricular cerebrospinal fluid. Not only cannot the actual brain-intracranial-fluid ratio be determined with accuracy in the living individual, but the ten-minute nitrous oxide ratio of fluid to blood cannot be more than roughly approximated because of nonhomogeneity of the solute concentration throughout the intracranial cerebrospinal fluid.

Further work is in progress to investigate the observations that the nitrous oxide concentration rises faster in the cisternal than in the ventricular fluid.

SUMMARY AND CONCLUSIONS

Techniques for obtaining intracranial cerebrospinal fluid through the jugular foramen are described.

During the cerebral blood flow determination by the nitrous oxide method, appreciable quantities of the gas appear in the cerebrospinal fluid, to a greater extent in the intracranial subarachnoid space than in the ventricles or lumbar sac.

It is probable that in most subjects the cerebral blood flow on the above basis is only very slightly (about 4 to 6 per cent) higher than indicated by the nitrous oxide method. In the presence of external hydrocephalus and cortical atrophy without ventricular dilatation, the true cerebral blood flow might be more significantly in excess of the value indicated by the nitrous oxide procedure.

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NORMAL DISTRIBUTION OF SERUM PROTEIN LEVELS IN ACUTE POLIOMYELITIS PATIENTS

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INTRODUCTION

DATA on serum protein levels were obtained from poliomyelitis patients observed in various stages during an acute outbreak in 1950. Since the findings led to conclusions unlike those recently derived from results of a similar study elsewhere[1a, b, c, d] it appeared of interest to record them. The implications of these findings will be discussed.

METHODS

The analytical technique for the measurements of total protein and albumin in the serum was that of Howe.[2] Although errors inherent to this technique are well known, the comparative nature of the data tended to yield a systematic error of minor significance.

RESULTS

A total of 283 determinations of serum protein levels were made in 125 of 224 patients admitted. Results were evaluated from two different standpoints: (a) temporal phase of and (b) degree of severity of the illness. Temporally, data were arranged and compared in four periods: 1 to 6; 7 to 12;

TABLE I. SERUM PROTEIN AND ALBUMIN LEVELS IN ACUTE POLIOMYELITIS PATIENTS ACCORDING TO VARYING SEVERITY AND DURATION

GROUPS	NO. OF DETERMINATIONS	TOTAL PROTEIN*			ALBUMIN*		
		MEAN	S.D.†	S.E.‡	MEAN	S.D.†	S.E.‡
A. By Severity							
Mild	70	7.04	1.50	0.18	4.66	0.90	0.10
Moderate	156	7.04	1.10	0.08	4.65	0.85	0.06
Severe	57	6.91	0.26	0.03	4.48	0.71	0.09
B. By Duration (Days of illness)							
1-6	38	7.15	1.12	0.18	4.72	0.83	0.13
7-12	90	7.20	0.18	0.02	4.71	0.44	0.04
13-18	59	7.19	0.87	0.11	4.71	0.59	0.08
19-84	96	6.87	0.90	0.09	4.61	0.56	0.06
Total	283	6.98	0.92	0.05	4.67	0.70	0.04

*All values are expressed as grams per 100 ml. of serum.
†Standard deviation.
‡Standard error.

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13 to 18; and 19 to 84 days after onset of initial symptom (not necessarily paralysis). Severity of each patient's illness was categorized as (1) mild, (2) moderate, (3) severe; employing Bower's criteria.[1a] A comparison of data from each category was made.

Table I shows mean values, with their standard deviations and errors, of serum protein and albumin levels of the 125 patients observed. The data comprise a total of 283 determinations in patients categorized, both as to severity and duration of disease. An inspection of the table reveals close similarity of results among all groups listed. In the group designated by severity, mean values of total protein were practically identical in all subgroups varying from 6.91 to 7.04 Gm. per 100 millimeter. Those of serum albumin were slightly, but not significantly lower in the "severe" than in the remaining groups.

In the group listed according to temporal phase of illness, mean values for serum protein and albumin were about constant, 7.15 to 7.20, and 4.71 to 4.72 Gm. per 100 ml., respectively, for the first two and one-half weeks of the disease. Both values were slightly low during the convalescent or chronic stage (19 to 84 days).

TABLE II. MEAN DIFFERENCES AND "P" VALUES FOR SERUM PROTEIN AND ALBUMIN LEVELS IN ACUTE POLIOMYELITIS PATIENTS ACCORDING TO VARYING SEVERITY AND DURATION

GROUPS	TOTAL PROTEIN		ALBUMIN	
	M.D.*	P.†	M.D.*	P.†
A. By Severity				
Mild vs. moderate	0.01	0.98	0.01	0.38
Mild vs. severe	0.13	0.14	0.18	0.20
B. By Duration				
(Days of illness)				
1-6 vs. 7-12	0.05	0.78	0.02	0.92
7-12 vs. 13-18	0.01	0.93	0.002	0.93
13-18 vs. 19-84	0.32	0.03	0.09	0.92

*Mean difference.
†Probability value calculated.

Table II shows the mean differences, with their calculated probability values (P), of serum protein and albumin levels among the subgroups designated in Table I. A "P" value of less than 0.05, indicating that there are less than five chances in a hundred that the observed differences could have been due to chance alone, is taken as significant. With one exception, all of the mean differences could be explained by chance phenomena. The exceptional group, those patients in the chronic stage of the disease, had lower mean values of total protein than acutely ill patients. The "P" value for this group versus that of the 13- to 18-day group, showed odds of less than 3 in 100 that the difference could have been due to chance. The analysis failed to reveal statistically significant differences in albumin levels among any of the groups tested.

Serial changes of serum protein and albumin concentrations in individual patients, not presented, followed no consistent pattern; such changes were of small magnitude (mostly less than 1.0 Gm. per cent), some increasing and some decreasing over the admission values.

DISCUSSION

Data of the present communication showed normal frequency distributions, without significant differences, for values of serum proteins during various grades and stages of acute poliomyelitis. Small but statistically significant decreases of protein levels were found only in the chronic stages of the disease. Such decreases may be explained in part by nitrogen deficits associated with the prolonged immobilization of convalescence.[3, 4]

Bower and his colleagues[1a, b, c, d] have reported hypoalbuminemia in acute poliomyelitis. Their data show decreases of 0.04 to 1.0 Gm. of serum albumin levels mostly between the seventh and tenth days of disease. They postulated a correlation between severity of disease and degree of lowering of serum albumin. In addition, they reported that plasma infusions resulted in increased levels of serum albumin effectuating removal of edema from the spinal cord with clinical improvement.

The interrelations between serum protein concentration and edema formation should be reviewed critically.

Starling's fundamental hypothesis explains net transfers of fluid between capillary and interstitial spaces as a result of imbalances between osmotic pressures of plasma and interstitial fluid.[5] Although able to account for the absorption of isotonic fluids injected into connective tissue spaces, it does not actually propose any mechanism of "hypo-proteinemic edema." Predictions of such a mechanism would be: (1) increased transudation with resulting decrease in plasma volume, and hence (2) a rise in concentration of serum protein until a new equilibrium is attained between the forces of transudation and absorption.[6] Adequate plasma volume measurements in poliomyelitis are not available at present, to our knowledge. From extensive histopathologic studies of human and experimental poliomyelitis, Bodian states that there "is no evidence that edema plays a dominant role in the production of paralysis."[7]

Whether or not infusions of plasma or albumin can indeed alter serum protein levels (except instantaneously) is open to question. Studies have indicated that plasma and albumin infusions are not efficacious in restoring to normal the albumin levels of hypoproteinemic patients.[8-10] On the other hand, high protein diets, if acceptable and assimilated, have been successful in correcting hypoproteinemia of malnutrition.[11a, b] It may be assumed that any decrease in serum protein level of poliomyelitis is associated with a generalized protein depletion.

It is well known[11a, b, 12] that each gram deficit of circulating albumin is equivalent to 30 Gm. deficit of tissue albumin. In a 70 kg. man (average plasma volume = 3.5 liters), 1 Gm. lowering of albumin per 100 ml. means 35 Gm. shortage of circulating albumin and $35 \times 30 = 1{,}050$ Gm. shortage in the tissues. The total deficit would be 1,085 Gm. Therapeutic replacement would require infusions of plasma of the order of magnitude of fifteen and one-half liters of plasma for a 1.0 Gm. per cent deficit in such a patient, assuming 100 per cent retention of nitrogen.

SUMMARY

A statistical study of 283 determinations of serum proteins on 125 patients with acute anterior poliomyelitis indicated normal frequency distributions for values of serum protein and albumin. No significant differences were found among patients of varying grades of clinical severity during their first three weeks of disease. Slight but significant differences of serum protein, found only in the chronic stage, presumably were associated with prolonged immobilization.

The relationships between serum proteins and edema formation together with effects of parenteral protein therapy are discussed.

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DEVELOPMENT OF RESISTANCE TO 4-ACETYLAMINOBENZALDEHYDE THIOSEMICARBAZONE BY MYCOBACTERIUM TUBERCULOSIS

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STREPTOMYCIN is at present the most useful agent available for chemotherapy in tuberculosis. Two serious disadvantages attend its employment, however, that is, toxicity to the patient and the acquisition of resistance to the drug by the microorganism. Reports of the efficacy of each new agent in the treatment of this disease are therefore attended eagerly, with the hope that one of them may prove at least as effective therapeutically without the undesirable effects accompanying the use of streptomycin.

The thiosemicarbazones, developed in Germany by Behnisch, Mietzsch, and Schmidt[1] in collaboration with Domagk,[2] have been subjected to clinical trial with some success in that country. Following the recommendations of Hinshaw and McDermott,[3] the Veterans Administration, Army, and Navy undertook a study of toxicity to human beings and of the emergence of bacillary resistance following the administration of 4-acetylaminobenzaldehyde thiosemicarbazone. The present report constitutes studies on the acquisition of resistance of tubercle bacilli to this drug as observed in one of the study units undertaking this project. Toxicity and clinical studies on this group of patients have been reported elsewhere by Shamaskin and associates.[4]

METHODS

Thirteen cases of pulmonary tuberculosis were treated. Seven had received previous courses of streptomycin either alone or with p-aminosalicylic acid and were treated with only the thiosemicarbazone.* The protocol called for daily doses of 50 mg. for one week, 100 mg. for one week, then 200 mg. until the end of the course of four months of treatment (Group I). The remaining six cases had had no previous chemotherapy, and were given daily doses of 1 Gm. of streptomycin in addition to tibione (Group II).

Twenty-four-hour sputum samples were collected before treatment, at two-week intervals during treatment, and at monthly intervals for four months after treatment was discontinued. Trisodium phosphate was used for digestion. Tubercle bacilli were recovered on one or all of three types of culture medium: inspissated potato egg-yolk of the American Trudeau Society, Petragnani's medium, or an egg-yolk agar. "Indirect" or subculture tests were made according to the tentative method recommended by the Laboratory Com-

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TABLE I. RESISTANCE OF MYCOBACTERIUM TUBERCULOSIS TO 4-ACETYLAMINOBENZALDEHYDE THIOSEMICARBAZONE

GROUP	PATIENT	LENGTH OF RX	PROPYLENE GLYCOL CONCENTRATIONS IN MEDIUM							
			3 PER CENT (ALL DRUG CONCENTRATIONS)			0.6 PER CENT IN 10 γ			3 PER CENT IN 50 γ	
			SENSITIVITY*	GROWTH		TIME AFTER RX START	SENSITIVITY†	GROWTH		TIME AFTER RX START
				TB-1	P.G.‡	WK. TO MO. POST.§		TB-1	P. G.‡	WK. TO MO. POST.§
TB-1 only	J.F.	10 wk.	R	50γ-col. to 1+	1+ to 3+	8 to 4 incl.	R	50γ-1+	2+ to 4+	6 to 2 incl.
	A.R.	4 mo.	R	1γ-1+	20 c. to 3+	14 to 2 incl.	R	10γ-2+	3+ to 4+	6 to 4 incl.
	L.M.	12 wk.	R	1γ-1+	2+	– – 3	R	10γ-1+	3+ to 4+	6 to 4 incl.
	I. W.	4 mo.	R	1γ-1+	3+	– – 1 to 3 incl.	R	10γ-2+-3+	3+ to 4+	10 to 4 incl.
I	J. K.	4 wk.	S	– – –	2+ to 3+	2 & 4 ‖ – – – –	R	10γ-1+	3+ to 4+	2 & 4‖ –
	A.H.	8½ wk.	S	– – –	3+	8‖ – – – –	R	10γ-1+	4+	8‖ –
	W.C.	5½ wk.	S	– – –	30 c.	5½ ¶ – – – –	S	–	4+	5½ ¶
II TB-1 and Sm	F.M.	4 mo.	R	50γ-1+	2+ to 3+	– 1 to 4 incl.	R	50γ-1+	3+ to 4+	14 to 1 incl.
	W.F.	3 mo.	R	1γ-30 c. to 1+	3+	4	R	10γ-1+	3+ to 4+	6 to 3 incl.
	A.D.	4 mo.	R	1γ-4 to 20 c.	3+	2 to 3 incl.	R	10γ-1+	3+	– 1
	H.P.	4 mo.	S	– – –	1+ to 3+	Through 4 mo. post.§	S	– – –	3+ to 4+	Through 4 mo. post.§
	W.P.	10½ wk.	S	– – –	0 to 2+	Through 14 wks.‖	S	– – –	3+ to 4+	Through 14 wk.‖
	P.B.	4 mo.	S	No growth till 3 mo. post.§ then S.			S	No growth till 3 mo. post.§ then S		

*R = growth on 1 γ or more, S = no growth on 1 γ or more TB-1 per ml. medium.
†P.G. = 1+ growth or more on 10 γ, S = less than 100 colonies on 10 γ TB-1 per ml. medium.
‡P.G. = Growth on propylene glycol control.
§Post. = After cessation of treatment.
‖ = Expired.
¶ = Drug discontinued.

mittee of the Veterans Administration Streptomycin Committee, using A.T.S. medium, with 30 per cent solvent for tibione in order that results might be comparable to those from other units. Final concentrations of drug were 50, 10, 5 and 1 γ per milliliter of medium and of solvent, 3 per cent. Inocula were Tween-albumin medium subcultures standardized by Hopkins tube measurements (0.1 mg. in 0.1 ml.).

Three per cent propylene glycol without the drug proved inhibitory to the growth of freshly isolated bacilli in varying degrees. Less than 1 per cent glycol had no inhibitory effect. Therefore the entire series of cultures from each patient was also tested on medium with less solvent (0.6 per cent or less except in medium containing 50 mg. of drug, which required 3 per cent glycol for solution). Proper solvent controls (with no drug) were inoculated with each test culture.

In the absence of accepted standards, criteria of resistance were arbitrarily established on the basis of growth of fourteen pretreatment cultures including all treated patients and an additional individual.

RESULTS

Pretreatment Cultures.—None of the fourteen grew on any concentration of thiosemicarbazone in the presence of 3 per cent glycol. Growth on 3 per cent solvent controls (no drug) was in general meager compared with growth on medium containing neither solvent nor drug.

With less solvent, some growth occurred in the presence of tibione. Thirteen of the fourteen cultures produce from a few colonies to 2+ growth on 1 γ per milliliter; nine of the fourteen had from 1 to 19 colonies on 5 γ and on 10 γ (0.6 per cent glycol), eight cultures grew slightly (from 1 to 16 colonies).

On the basis of these results, the following arbitrary criteria for thiosemicarbazone resistance of bacilli were adopted: with 3 per cent glycol, growth on 1 γ or more per milliliter of medium; with 0.6 per cent glycol, 100 colonies or more on 10 γ per ml.

TB-1 Resistance During the Following Treatment.—Results are presented in Table I. Bacilli resistant to Tibione according to our standards were recovered from the same patients with both solvent concentrations provided treatment was continued over a sufficiently long period (Table I: first four patients of Group I and first three patients of Group II). It is evident from the table that tibione resistance was detected earlier in the course of treatment when the test medium contained less than 1 per cent solvent. Thus on the latter medium, bacilli considered resistant were recovered from two patients of Group I (J. K. and A. H.) at two and eight weeks, respectively, although there was no evidence of resistance if medium contained 3 per cent glycol. Similarly, using the lesser amount of solvent, three more of Group I were excreting resistant bacilli at six weeks and one at ten weeks, but with 3 per cent solvent, eight and fourteen weeks of treatment, respectively, were given before detection of resistant bacilli in two cases, and the full course (twelve and sixteen weeks) for the latter two cases. The seventh patient of this group, who received only five and one-half weeks of treatment, apparently excreted only sensitive bacilli (both solvent concentrations).

A similar delay in detection of resistance in the presence of 3 per cent glycol occurred in Group II. Resistant bacilli were eventually recovered from the same cases in this group on both solvent concentrations, however.

The number of cases is admittedly small, but comparison of results from Groups I and II indicates that the simultaneous administration of streptomycin and tibione lessened the incidence of tibione-resistant bacilli.

Tubercle bacilli capable of growing in the presence of 50 γ tibione per milliliter were recovered from one patient in each group in spite of the presence of 3 per cent glycol.

Streptomycin Resistance.—The administration of tibione to patients excreting bacilli resistant to streptomycin as a result of previous chemotherapy appeared to have no influence on streptomycin resistance (Table II, first four patients). When both drugs were given, the incidence as well as the time of emergence of streptomycin-resistance was similar to that which occurs following streptomycin only (Table II).

TABLE II. STREPTOMYCIN SENSITIVITY

Group I: Thiosemicarbazone Alone (previous Sm).

Group II: Streptomycin and Thiosemicarbazone (no previous chemotherapy).

GROUP	PATIENT	LENGTH OF R	SM SENSITIVITY	TIME AFTER R START		
				WKS.		MO. POST. R
I TB-1 only	I. W.	4 mo.	PR to SR	Pre	to	3 incl.
	A. R.	4 mo.	R	Pre	to	4 incl.
	L. M.	12 wk.	PR to R	Pre	to	4 incl.
	J. F.	10 wk.	R	Pre	to	4 incl.
	A. H.	8½ wk.	S	Pre – to 8 wk.		
	W. C.	5½ wk.	S	Pre – to 5½ wk.		
	J. K.	4 wk.	S	Pre – 4 wk.		
II Sm and TB-1	F. M.	4 mo.	PR to R	8	to	4 incl.
	A. D.	4 mo.	PR	10	to	4 incl.
	H. P.	4 mo.	PR and R			3 & 4
	P. B.	4 mo.	S	Pre		
			No growth	2	to	2 incl.
			R			3 (1 col.)
	W. F.	3 mo.	SR, PR, R	10	to	4 incl.
	W. P.	10½ wk.	R	6	to	10 incl.

SR = Slightly resistant, small amount of growth on 10 γ streptomycin per milliliter.
PR = Partially resistant, less growth on 10 γ than on control.
R = Resistant, growth on 10 γ and control approximately equal.

DISCUSSION

Russell, Bush, and Hurwitz[6] also found propylene glycol in concentrations of more than 1 per cent inhibitory to the growth of tubercle bacilli. Using 1 per cent glycol, however, their results with cultures previously unexposed to tibione were similar to those obtained in the present work, since bacilli from all five sputum concentrates tested grew on A.T.S. medium containing 1 γ tibione and 1 per cent glycol and from only one of the five cases, on 5 γ and 10 γ (3 and 40 colonies, respectively). In the present report, all cultures considered resistant produced at least 1+ growth in the presence of 10 γ.

Davis and Schwartz, using antipyrine as solvent, did not present results with pretreatment cultures, but noted bacilli resistant to 50 γ tibione as early as four weeks after the beginning of treatment.

In the present report, eventual recovery of tibione-resistant organisms from the same cases on both glycol concentrations when treatment was given over a

sufficiently long period, supports the contention that acquired resistance to the drug is being observed. Apparent suppression of the development of tibione resistance by simultaneous treatment with streptomycin and the absence of indications that tibione suppresses the development of streptomycin resistance might offer a clue to the mechanism of action of these two antibacterial substances. It might also indicate that tibione resistance is less stable than streptomycin resistance, an interpretation which possibly has some support in the data in Table I. It will be noted that two to four months after treatment was stopped, tibione-resistant bacilli were not isolated from certain cases from which they had been previously repeatedly recovered.

The importance to the patient of the degree of thiosemicarbazone resistance noted in the present studies is not apparent. The fact that less growth occurred on tibione medium than on corresponding solvent controls would indicate that many bacilli in each culture were still sensitive to the drug. Theoretically, treatment should benefit a patient as long as sensitive bacilli are present. The importance of determining when resistance is sufficient to make a drug ineffective in treatment has been previously pointed out.[8] Only a careful correlation of clinical response with sensitivity test results will answer this question.

SUMMARY

Arbitrary standards of tibione resistance of cultures recovered from groups of patients treated with (a) tibione only, and (b) tibione and streptomycin have been set up on the basis of growth of pretreatment cultures on tibione-containing media.

Tubercle bacilli resistant to 50 γ tibione per milliliter culture medium were recovered from two of thirteen cases. Bacilli resistant to at least 10 γ per milliliter were recovered from eight of thirteen cases at two to fourteen weeks after beginning therapy, and from an additional case one month after treatment was stopped.

Although the number of cases is admittedly small, results suggest that simultaneous administration of streptomycin and tibione delayed the emergence and lessened the incidence of tibione-resistant bacilli. There is no indication that either time of emergence or incidence of streptomycin-resistant bacilli was influenced.

Bacilli from four tuberculous patients resistant to streptomycin as a result of previous chemotherapy underwent no significant changes in resistance to streptomycin during or following exposure to tibione.

Inclusion of 3 per cent propylene glycol in test medium inhibited in varying degrees the growth of tubercle bacilli and also delayed recognition of tibione-resistant bacilli. Less than 1 per cent propylene glycol appeared to have a negligible effect on growth.

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EXPERIMENTAL STUDY OF WOUND HEALING

II. Effects of Various Substances on Wound Healing

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WE HAVE described previously a technique for studying wound healing in the guinea pig, and we have reported faster healing of wounds following application of detergents and of an antacid.[1] This study has been extended to the use of a number of other biological substances which might affect wound healing.

We refer to a previous paper for mechanisms of healing of skin lesions and the effects of various substances on the healing process.[1]

METHODS

The method has been described in our previous paper.[1] Guinea pigs of both sexes were used, weighing between 250 and 300 grams. Under anesthesia and with aseptic precautions, two wounds (in a few experiments one wound) each measuring 1 sq. cm. in area were produced on the saddle area of both flanks. The skin was dissected out cleanly with scissors, down to the subcutis, and a flat lucite ring was glued tightly to the skin surrounding each wound. In case a local agent was to be tested, it was applied to one wound only. Then a piece of transparent x-ray film was glued on top of the lucite ring. Through the film, the rate of healing could be observed. The area of the wound was traced on celluloid, and it was measured with a planimeter. The lucite ring was glued on so that it surrounded the wound closely, and it prevented largely the contraction phase of wound healing. In this way, we expected to be able to observe and measure the process of healing without the complication of contraction of the wound, which usually is uneven, and which in our experience often obscures actual healing. Also, we thought that, in order to analyze wound healing, the simplest method would be the one which excluded complicating factors. Animals that were sick or which died during the period of the experiment were excluded from the test series.

Wound healing was expressed as the size of the healed area in per cent of the size of the original wound. Healing was considered to be complete when the wound had healed 95 per cent or more. The day of the operation was counted as the first postoperative day. The number of days for 80 per cent healing of the wounds were compared, because we had found earlier that this point gave significant information about the progress of healing.[1] All data were evaluated statistically.* Antireticulocytic cytotoxic serum was prepared according to the method of Movitz and associates.[2]†

RESULTS

The results are summarized in Table I. It is seen that the present control series yielded values similar to those obtained earlier.[1]

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TABLE I. SUMMARY OF RESULTS

PROCEDURE	NO. OF GUINEA PIGS	TOTAL NO. OF WOUNDS	AVERAGE NO. OF DAYS FOR HEALING		ON 8TH DAY			
			100%	80%	RANGE OF % HEALING	MEAN OF % HEALING	NO. OF WOUNDS 80% HEALED OVER TOTAL NO. OF WOUNDS	NO. OF WOUNDS WITH 100% HEALING
Controls	37	45	12.5	10.5	35-90	65	2:45	0
Controls 1948[1]	20	35	11.7	10.5	19-100	49	1:35	1
ACS 0.125 ml., 1:10 dilution, s.c.	12	12	8.9	8.1	65-80	78	11:12	0
ACS 0.1 ml., 1:100 dilution, s.c.	8	8	8.7	7.9	75-96	85	6:8	2
ACS 0.1 ml., 1:1000 dilution, s.c.	10	10	9.3	8.2	75-80	79	9:10	0
Normal Rabbit Serum 0.125 ml., 1:10 dilution, s.c.	12	24	11.9	10.3	40-70	53	0:24	0
Enterogastrone 50 mg. s.c.	4	6	14.4	10.7	59-68	62	0:6	0
Urogastrone 20 mg. s.c.	5	10	13.3	11.5	58-81	69	0:10	0
Rutin 25 mg. on wound	5	5	13.2	10.5	40-76	59	0:5	0
Ascorbic Acid 25 mg. on wound	4	4	14.0	11.6	40-76	61	0:4	0
Desoxycorticosterone acetate 0.5 mg. s.c.	4	8	13.3	10.0	51-70	60	0:8	0
Glutathione 25 mg. s.c.	5	10	12.9	10.4	58-81	69	1:10	0
Colchicine 0.65 mg. s.c.	5	10	13.5	11.4	37-60	51	0:10	0
Aluminum foil on wound	11	11	11.4	11.0	40-80	66	3:11	0

Of all substances tested, only antireticulocytic cytotoxic serum (ACS) enhanced wound healing. This is expressed particularly by the number of wounds which had healed 80 per cent on the eighth postoperative day. Also, 100 per cent healing was faster than in the controls. A control series to the three tests with ACS was performed with normal rabbit's serum, and it is seen that the data resemble control values closely. The mean healing times were 8.92 ± 0.09; 8.69 ± 0.21; 9.30 ± 0.22 days, respectively, for 1:10, 1:100, and 1:1000 dilutions of ACS. When the results of the three groups are compared with those of the controls, the probability is well below 1 per cent that these differences could have occurred by chance alone. Statistically, the three ACS treated groups did not differ among themselves. The validity of the difference is even enhanced when the results in the ACS series are compared with the results of the series treated with normal rabbit serum.

Enterogastrone, urogastrone, rutin, ascorbic acid, desoxycorticosterone, glutathione, colchicine, and aluminum foil had no accelerating effect on the rate of wound healing. In the experiments with aluminum foil applied locally to the wound, the number of wounds 80 per cent healed on the eighth day were somewhat greater than in the control series, but the rest of the data did not indicate any significant difference from the controls. In one-half of the tests the shining side and in the other half of the tests the dull side of the aluminum foil was put on the wound. The results indicated no difference between the two procedures.

A number of other substances were tested, but not included in Table I. These were preparations of blood, of plasma, and tantalum oxide, all applied locally to one wound. None of the data showed significant variation from the control values.

Like in our previous publication,[1] we observed that in a number of experiments, relaxation of the wound occurred. By relaxation we understand that instead of getting smaller as time advanced, there would be a point when the wound enlarged. In a few experiments this occurred twice in the same animal. Observations on relaxation were not made until the second postoperative day.

The number of guinea pigs used for analysis of relaxation was 147, with a total number of 160 wounds. Relaxation was observed in 55 of the 147 guinea pigs, i.e., in 37 per cent. The 55 guinea pigs had 61 wounds, which means that relaxation was observed in 38 per cent of all wounds. In a few instances, both wounds in the same animal relaxed (three animals), more frequently however only one of the two wounds relaxed (55 animals). The degree of relaxation in per cent in the entire series of 160 wounds varied between 2 and 50 per cent, with an average degree of relaxation per wound of 3 per cent.

Table II indicates the time of occurrence of relaxation, and it is seen that

TABLE II. INCIDENCE IN TIME OF RELAXATION

DAYS POSTOPERATIVELY	NO. OF WOUNDS
2-4	14
5-9	28
10-12	19

TABLE III. INCIDENCE IN PER CENT OF RELAXATION OF WOUNDS

PER CENT OF RELAXATION	NO. OF WOUNDS
2-4	18
5-9	17
10-14	11
15-19	8
20-25	4
30-50	3

it is distributed from two to twelve days postoperatively, with its greatest incidence five to nine days postoperatively. The degree of relaxation is demonstrated in Table III. It is seen that in most animals there was 2 to 14 per cent relaxation, but that relaxation up to 50 per cent occurred in a few wounds.

DISCUSSION AND CONCLUSIONS

We are not able to explain to what the beneficial effects of the antireticulocytic cytotoxic serum may be due. It may have something to do with prevention of excessive granulation in the crater of the wound which would facilitate epithelization.[1] The experiments with normal rabbit serum demonstrated that the accelerating effect of ACS on wound healing was not a property of normal serum.

In using enterogastrone and urogastrone, we did not presume that healing of skin wounds and of peptic ulcer are identical processes. However, healing of both types of wounds has so much in common that we considered it possible that substances which help in the epithelization of wounds in the stomach or duodenum might also help in the new formation of epithelium of the skin. Healing of peptic ulcer is not only by epithelization, but also by filling of the ulcer crater with fibrous tissues, similar to the healing of skin defects. The negative effect of enterogastrone and urogastrone on the healing of skin wounds parallels their negative effect in the treatment of peptic ulcers reported by a number of investigators.

Rutin and ascorbic acid were applied locally. It is possible that they irritated the wound chemically and that, if they had been given by injection, different results might have been obtained.

If slowing up of mitosis would produce a change in wound healing, then results of experiments with colchicine would contribute to the analysis of the healing process. However, the effect of colchicine on wound healing was not marked. It is possible that a slight degree of inhibition occurred, but our data do not show a significant difference to the controls. Thus, our results seem to support the theory advanced by others and discussed in our previous communication, that wound healing occurs not so much by mitotic processes, but by migration of cells from the borders of the wound.[1]

The observations on relaxation of wounds are puzzling. It is conceivable that the operative trauma can produce a contraction of the tissues underlying and surrounding the wound and that, as this irritation disappears, the wound may relax. However, this cannot explain that relaxation of the wound was observed up to twelve days following the operation, that it occurred usually only

on one side and not on both sides, and that it did not occur in most of the animals. It is possible that relaxation has something to do with the wound healing process itself. During the first seven or eight days the healing process in the controls was observed to be rather irregular, and it began to show regular progression only after the seventh or eighth day. This was the reason why we chose the eighth day as a cutting point in the evaluation of the data on healing. We do not know what biological significance relaxation of wounds may have. No correlation between the condition of the animal and relaxation of a wound could be found.

Faster healing of a wound need not necessarily mean better healing. However, if the healing process can be speeded up and yet be normal and complete, then faster healing of wounds would be advantageous. Histologic studies and measurement of tensile strength of such wounds may answer this question. Grossly, there was no difference detectable between wounds that healed fast and those that had healed slowly.

SUMMARY

The effect of antireticulocytic cytotoxic serum, enterogastrone, urogastrone, rutin, ascorbic acid, desoxycorticosterone, colchicine, glutathione, aluminum foil, blood, plasma, and tantalum oxide on healing of experimental skin wounds in the guinea pig was investigated.

Of these substances only the antireticulocytic cytotoxic serum seemed to produce a faster rate of healing of experimental wounds.

In a relatively large number of wounds, relaxation of the wound was observed.

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EVALUATION OF THE WRIGHT AND SUPRAVITAL STAINING METHODS IN INFECTIONS WITH MONONUCLEAR CELL RESPONSE

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PRELIMINARY observations have indicated that in simultaneously taken blood samples the Supravital staining method yielded significantly higher monocyte counts than the Wright technique. Although such discrepancies could have been due to simple errors in method, it is possible that a more fundamental reason is involved. Only two previous reports have been found dealing with this problem, but their primary concern was in the variance of the count of the polymorphonuclear cells.[1, 2] The purpose of this study was to assess objectively the difference between the two methods using a sufficiently large number of samples to be statistically significant without entering, however, into the still prevailing controversy that inspired the original proponents of these two techniques.[3]

METHODS

In performing the Supravital staining, the detailed description by Doan and Ralph[4] was followed. The dye concentration (a mixture of Neutral Red and Janus Green) was properly standardized as recommended for human and rabbit blood. The conventional Wright's modification of the Romanowsky tri-acid stain* for fixed films of peripheral blood was employed. For human blood samples, cover slip preparations were used, and slide preparations were employed for rabbit differentials. To obtain more reliable averages, a minimum of three hundred to five hundred cells was counted with each method. In all, one hundred thirty-five human cases and sixty-three rabbits were studied, and the results were statistically analyzed. In the tables and chart, only the findings relative to the polymorphonuclear leukocytes (PMN), lymphocytes (Ly) and monocytes (Mo) were tabulated. The deviation in the eosinophile and basophile counts was too small to be included in this chart. The Ly-s, usually segregated according to their variable size, were grouped together.

RESULTS

(A) *Human Counts.*—The one hundred thirty-five samples were obtained from: (1) 100 normal young female adults tested in connection with a student-nurse health program. (2) Twenty-five patients having severe pulmonary tuberculosis. These patients were all inmates of tuberculosis wards where the advanced cases with positive sputum were congregated. However, no selection was made according to their actual clinical status. (3) Ten patients with well-established infectious mononucleosis. The clinical diagnoses in these cases were confirmed by the heterophile agglutination test as described by Davidsohn,[5] and by the hematologic findings.

The results as seen in Table I can be correlated as follows: in the samples from the normal student nurses (1), the shift in the qualitative count was not constant with the two methods. In the majority of cases, the Supravital method

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*Todd, I. C., and Sanford, A. H.: Clinical Diagnosis by Laboratory Methods, W. B. Saunders Co., Philadelphia, 1948, p. 233.

TABLE I. WHITE BLOOD CELL COUNTS USING SUPRAVITAL AND WRIGHT STAINING METHODS IN HUMAN BEINGS

SOURCE	CELL TYPES	FREQUENCY OF DEVIATION*	TOTAL NUMBER OF CELLS PER CU. MM. AND PER CENT						$\sigma\bar{x}$	"t"
			SUPRAVITAL			WRIGHT				
			AVERAGE	PER CENT	MIN.-MAX.	AVERAGE	PER CENT	MIN.-MAX.		
1. Normal female adults (100) Av. Total WBC: 7062 (4100-10900)	PMN	S = W 6 S > W 59 S < W 35	4559	63.6	1932-9234	4426	61.7	1596-8424	—	—
	Ly	S = W 3 S > W 12 S < W 85	1594	23.2	720-3080	1946	28.3	955-3666	0.45	11.0
	Mo	S = W 11 S > W 76 S < W 13	685	9.6	219-1491	494	7.2	240-1157	0.28	8.2
2. Patients with tuberculosis (25) Av. Total WBC: 12110 (4750-22400)	PMN	S > W 15 S < W 10	8767	70.3	2883-17539	8537	68.3	2755-16800	—	—
	Ly	S > W 1 S < W 24	1212	11.3	416-2540	2139	19.5	423-4290	1.14	7.2
	Mo	S > W 25 S < W 0	1474	12.8	666-2867	543	4.7	172-1318	0.55	15.0
3. Patients with infectious mononucleosis (10) Av. Total WBC: 14635 (5900-34050)	PMN	S > W 9 S < W 1	3481	25.3	1475-8768	2844	21.0	1398-7576	—	—
	Ly	S > W 0 S < W 10	8556	59.9	3068-15877	10610	70.9	13876-23750	1.02	7.8
	Mo	S > W 10 S < W 0	1440	11.3	902-2322	399	3.3	221-586	1.75	6.3

*S = Supravital
W = Wright
The direction of deviation between the two stains and the number of cases in which such deviation occurred is shown in column three.

yielded somewhat higher numbers of PMN-s and Mo-s, whereas the Ly-s dominated more frequently in the Wright staining method. Although these actual differences between the averages of the two methods appeared slight, they still proved to be statistically significant (last column) because of the large number of tests (one hundred). On the whole, however, in normal cases, the two methods compared favorably with each other.

The differences between the two methods became more conspicuous under pathologic conditions. In tuberculosis (2), the greatest discrepancies occurred, as expected, in the Ly-s and Mo-s. The PMN leukocytes increased, as did the total white cell count, but this was recorded equally by both methods with only little difference between the two. Contrarily, the Ly-s and Mo-s showed pronounced reciprocal changes with the Supravital method; both the absolute as well as the relative number of Mo-s were increased, whereas that of the Ly-s was decreased, reversing the ratio between the two cell types. With the Wright staining method such changes were not observed. In fact, the absolute number of Mo-s remained practically the same as in the normal group; the Ly-s, on the other hand, exceeded the normal level. This explains why the Mo/Ly ratio stressed by workers using the Supravital technique has not been accepted generally by those employing the Wright stain. In our Supravital figures, this ratio rose from 0.4 (upper normal limit 0.5) to 1.2, whereas in the Wright preparations, it remained surprisingly constant, as low as 0.25.

In infectious mononucleosis (3), the discrepancy was somewhat different, affecting only the Mo-s. The number of PMN-s was reduced and that of the Ly-s was strikingly increased in both methods as it has been recorded by other workers. A difference was found in the Mo counts in so far as, in Supravitally stained preparations, their number was increased above normal while the Wright technique showed a decrease. This explains the conflicting reports regarding the percentage of Mo-s in the hematologic response of this disease.[6]

(B) *Rabbit Counts.*—Sixty-three samples were studied; these can be divided into three groups:

(1) Twenty-five originated from normal rabbits which were used as controls: (2) thirty from rabbits injected with *Histoplasma capsulatum**; (3) eight from rabbits injected with *Listerella monocytogenes* known to produce a monocytic response.

The findings are computed in Table II. It appears that in normal animals the qualitative distribution of cells followed about the same pattern in both methods, but, as in human beings, the number of PMN-s was somewhat higher with the Supravital and the number of Ly-s was increased with the Wright staining method. The absolute, as well as the relative, number of Mo-s was unquestionably higher with the Supravital technique. In experimental histoplasmosis, the results simulated somewhat those found in human tuberculosis. The PMN and Mo counts were higher with both methods but more so with the Supravital technique. In the case of the Ly-s, the Supravital figures showed a

*These animals also formed part of a separate investigation.

greater decline. Judging from the average count, the elevation of Mo-s was only moderate, but this was due to their great variation in the course of infection as described in a previous paper.[7]

In *L. monocytogenes* infections, the discrepancies between the two methods were analogous to those outlined for the former groups. With the Supravital

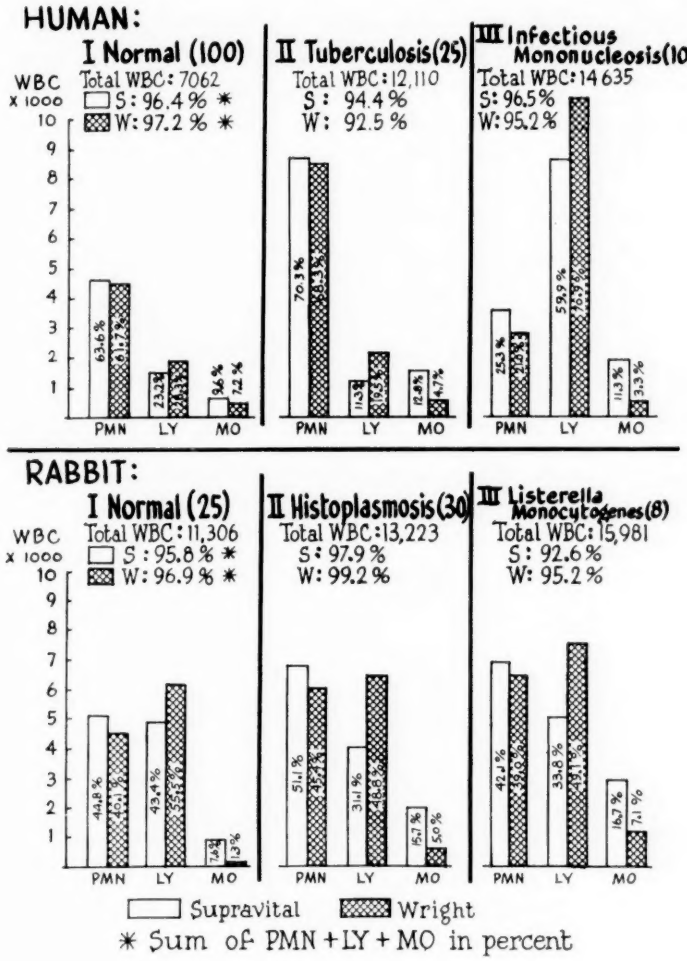

Fig. 1.

method the number of Ly-s was less and that of the Mo-s was more than with the Wright stain. The absolute increase in Mo-s was not as conspicuous in these animals as has been encountered by the authors. In previous experiments with different strains of this organism, Mo counts have been found up to 55 per cent. Such varied behavior is not unusual among different *Listerella* strains.

Fig. 1 illustrates the nature and trend of changes in all groups.

COMMENT

No simple explanation can be offered for the discrepancies between the two methods. The pros and cons have been set forth ably by the proponents of both methods. However, it may be pointed out again that because of the greater

TABLE II. WHITE BLOOD CELL COUNTS USING SUPRAVITAL AND WRIGHT STAINING METHODS IN RABBITS

SOURCE	CELL TYPES	FREQUENCY OF DEVIATION*	TOTAL NUMBER OF CELLS PER CU. MM. AND PER CENT						$\sigma_{\bar{x}}$	"t"
			SUPRAVITAL			WRIGHT				
			AVERAGE	PER CENT	MIN.-MAX.	AVERAGE	PER CENT	MIN.-MAX.		
1. Normal (25) Av. Total WBC: 11306 (7300-16750)	PMN	S > W 20 S < W 5	5118	44.8	2446-9186	4552	40.1	2111-10068	—	—
	Ly	S > W 3 S < W 22	4841	43.4	1443-9213	6254	55.5	2833-10636	1.58	7.6
	Mo	S > W 25 S < W 0	882	7.6	146-1656	157	1.3	24-406	0.58	10.8
2. Infected with *H. capsulatum* (30) Av. Total WBC: 13223 (7800-33500)	PMN	S > W 26 S < W 6	6825	51.1	3116-14593	6042	45.4	2770-14256	—	—
	Ly	S > W 1 S < W 29	4065	31.1	2018-15209	6353	48.8	2813-18861	1.59	11.2
	Mo	S > W 30 S < W 0	1977	15.7	575-4121	601	5.0	29-3015	0.93	11.6
3. Infected with *L. monocytogene* (8) Av. Total WBC: 15981 (10650-29050)	PMN	S > W 5 S < W 3	6889	42.1	4035-13857	6425	39.0	3238-13276	—	—
	Ly	S > W 0 S < W 8	5002	33.8	3728-6913	7483	49.1	5488-9964	2.92	5.2
	Mo	S > W 8 S < W 0	2880	16.7	1076-7756	1231	7.1	48-3399	1.65	5.9

*S = Supravital
W = Wright
The direction of deviation between the two stains and the number of cases in which such deviation occurred is shown in column three.

fragility of the neutrophilic granulocytes than of the lymphocytes, the percentage of neutrophils is lower in the Wright technique than in the Supravital, in which the mechanical injuries are minimal. Also, differential criteria between some monocytes and some intermediate lymphocytes are more sharply defined with the neutral red-Janus green staining of living cells than with Romanovsky's stain of fixed films. That the discrepancies are real and not due to inaccuracies of procedure has been verified by statistical analysis.* It could be shown that all the "t" values derived from the standard errors are significant at the 1 per cent level. On closer inspection it is quite evident that there exists a reciprocal interplay between the Ly and Mo counts which is reversed in the two methods. If the Ly-s are higher by one method, the corresponding figures for Mo-s are lower and vice versa. Although this holds true for both, the control as well as the pathological groups under study, the increase in Mo-s is more pronounced under pathologic conditions with the Supravital technique. This finding in Supravital preparations accounts for the elevation of the Mo/Ly ratio in tuberculosis of human beings. This elevation of the Mo/Ly ratio is absent when the Wright method is used. In infectious mononucleosis, both the Ly-s and Mo-s are reported to be increased when Supravitally stained, whereas by Wright staining only the Ly count is considered to be elevated. In experimental histoplasmosis of rabbits, serial blood examinations were performed by us and the monocytic response in the infected animals would have been missed completely if the Wright technique alone had been used. In *L. monocytogene* infection, using this particular strain, the monocytic response was only demonstrable by the Supravital technique and not at all by the Wright method.

CONCLUSION

Comparative studies with the Supravital and Wright staining methods revealed a significant difference between the results obtained by the two techniques. The monocytic elements are particularly affected by this discrepancy. It seems desirable that both methods should be carried out concurrently in questionable cases. The Supravital technique deserves a much wider application than is common today in hematological laboratories.

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THE IMPORTANCE OF THE SHEEP CELL SUSPENSION IN THE COMPLEMENT FIXATION REACTION: DESCRIPTION OF AN ACCURATE TECHNIQUE FOR THE PREPARATION OF A UNIFORM CELLULAR SUSPENSION

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INTRODUCTION

THE complement fixation test is an important laboratory procedure with an end point both obvious and discrete. The importance of standardizing reagents for this procedure[1] is emphasized, more particularly, with the recent employment of the 50 per cent hemolytic end point in the titration of complement[2-4] and in the complement fixation test for amebiasis. Here, more than minimal aberration from the standard cannot be tolerated, and conclusions are valid only if the laboratory is scrupulous in adhering to prescribed methods embracing not only the actual test techniques but also the preparation and standardization of reagents.[5]

Like any system of variables, the complement fixation test is subject, mathematically, to an error greater than the sum of the expected error in each of the variables. Utilized in the complement fixation reaction are five such variables, namely, antigen, hemolytic amboceptor, complement, sheep erythrocytes, and human serum. Furthermore, this test is unique in that the estimation of the strength of three of the reagents is determined upon a basis of a fourth variable which is also used directly in the test. The final step in the complement fixation test is actually a titration of the fifth variable, the human serum. The sheep cells, therefore, assume paramount importance not only in the test but also in the quantitative determination of the other variables, antigen, amboceptor, and complement, to be used in the test.

The role assigned the complement fixation tests in the establishment of a definitive diagnosis of syphilis has resulted in a minutely detailed procedure of preparation and titration of antigen,[6] complement,[7] and anti-sheep cell hemolysin,[8] as described in a standard textbook on the subject.[9] The sheep-cell suspension employed in complement fixation tests is unique in laboratory procedures. Not only is it used in the titration of the other four variables, but it is also used in both the bacteriolytic and hemolytic systems. It is therefore obvious that an accurate and reproducible determination of its concentration be made if uniform results are to be expected.

The sheep cells must be treated with discretion during the preparation of the suspension to prevent cellular alteration obviating the validity of the test. Such cellular alteration may be discerned in two ways. Actual destruction of

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the cell results in visible hemoiysis rendering the suspension useless. More serious because of impossibility of recognition prior to use of the suspension is a covert alteration of the exterior of the cell, insufficient to produce cellular disruption with resultant visible hemolysis, but of a sufficient degree to render the cell more susceptible to hemolysis when mixed with its antibody in the presence of complement. Obviously, this condition is not recognized unless sought in serial complement or amboceptor titrations.

Accurately prepared suspensions of sheep cells can be made by centrifuging the washed cells to minimum volume and adding sufficient saline to produce the desired concentration. Maximum cellular packing, however, occurs commonly only with the undesired feature of cellular damage.[10] This damage is usually not of sufficient magnitude to produce spectrophotometrically detectable hemolysis in the supernatant. However, there is alteration of the physicochemical qualities of the erythrocytes which renders them more susceptible to the action of the hemolytic amboceptor in the presence of the complement. This alteration, following the centrifugation of the cells to constant volume, can be demonstrated in three ways. Erythrocyte fragility studies determine the degree of resistance of these manipulated cells to hypotonic saline. Complement and amboceptor titration studies demonstrate the susceptibility of the manipulated cells to these two reagents.

METHODS AND MATERIALS

It is evident that the concentration must be accurately determined if uniform results are to be expected. Furthermore, accurate determination of the concentration must be effected in such a manner as to preclude the possibility of obvious or inapparent cellular damage. The method to be described fulfills these two criteria, and consists essentially of substitution of a portion of the cells for the total amount to be subjected to maximum packing in the centrifuge.

Thirty parts of fresh sheep blood not obtained under aseptic conditions are mixed with one part of the following solution:

Sodium citrate	8.0 Gm.
37 per cent formaldehyde	53.2 c.c.
0.9 per cent sodium chloride	800.0 c.c.

The blood mixed with the diluent is then centrifuged at 2,000 r.p.m. for ten minutes, after which the supernatant is aspirated and replaced by an equal volume of 0.9 per cent saline. This process is repeated four times, employing five-minute periods of centrifugation at 2,000 r.p.m. rendering the supernatant albumin free, after which 0.9 per cent saline is added to the cellular precipitate to make a suspension somewhat greater in concentration than the desired 2 per cent. After the suspension has been agitated to insure uniformity, a portion is aspirated to fill a Rourke-Ernstene[11] hematocrit tube which is then centrifuged at maximum speed for the time necessary to produce minimum cellular volume, the true hematocrit (H). The suspension from which the Rourke-Ernstene tube was filled is then placed in a graduate and the volume (V_1) noted. From the product of this figure and the true hematocrit divided by the desired percentage is subtracted the initial volume to give the amount of 0.9 per cent saline which must be added to the initial volume to make the suspension of desired concentration. The procedure may be expressed by the formulas:

$$(1)\quad X = \frac{V_1 H}{P} - V_1$$

$$(2)\quad V_c = X + V_1$$

where X = amount of 0.9 per cent NaCl to be added to initial volume to dilute to true suspension of desired concentration.
V_c = final total volume of suspension.
V_i = initial volume of suspension made slightly in excess of desired concentration.
H = true hematocrit.
P = desired percentage of suspension.

In this manner, true 2 per cent suspensions may be produced, the uniformity of which may be demonstrated by identical turbidimetric readings on the spectrophotometer. Equally important is the fact that the suspensions may be produced without subjecting the cells to be used to the damage incurred by excessive centrifugation.

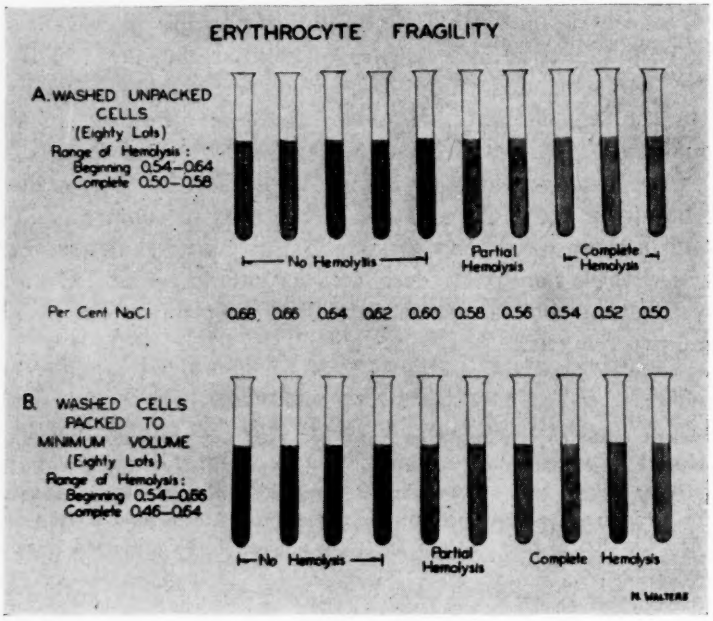

Fig. 1.

DISCUSSION

The procedure commonly employed in the preparation of a 2 per cent sheep-cell suspension as used in the Kolmer modification of the Wassermann technique results in one of two undesirable ends:

1. The production of a true 2 per cent suspension of sheep cells, not damaged sufficiently to result in detectable hemolysis prior to addition of amboceptor and complement, but altered in their physicochemical qualities so as to render them more susceptible to hemolysis as demonstrated by: (a) altered erythrocyte fragility, (b) altered complement titration, and (c) altered amboceptor titration.

2. The production of a suspension of sheep cells which is usually less than 2 per cent in concentration.

Cellular suspensions prepared by the technique here described are mathematically 2 per cent in concentration and are reproducible in identical concentration for daily use. Comparison of the cellular stability of the suspensions prepared by the new technique with those suspensions made from cells sub-

jected to the excessive manipulation incident to maximum centrifugation demonstrates that those cells subjected to a minimum of trauma are more stable, by three tests, than those traumatized by maximum centrifugation.

The three tests utilized in estimation of the stability of cells in suspension are erythrocyte fragility tests, complement, and amboceptor titrations. While it is recognized that sheep-cell suspensions utilized in complement fixation reactions are not submitted to hypotonic saline, it was felt that erythrocyte fragility studies would, nevertheless, give an indication of the strength of the cells.

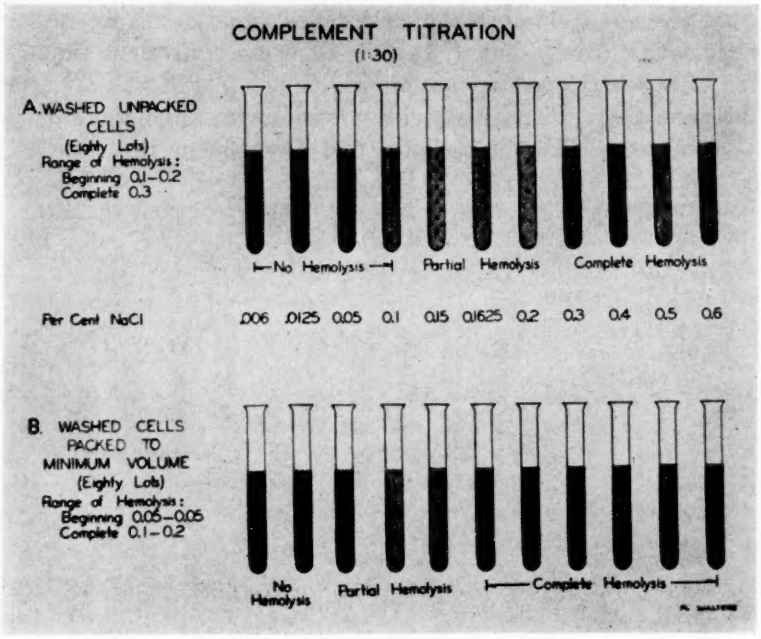

Fig. 2.

Fig. 1 demonstrates the average comparative resistance of eighty lots of cells to hypotonic saline. The upper row represents suspensions made by the new technique, while the lower row represents those prepared by the previously utilized procedure of maximum centrifugation. The decreased resistance of the latter group is shown schematically. Fig. 2 represents the susceptibility of the cells to hemolysis when varying amounts of complement are added to a constant volume of hemolytic amboceptor and cell suspension. The upper row of tubes demonstrates the greater resistance to hemolysis of cellular suspensions prepared by the new technique, while the lower row shows the markedly increased susceptibility to hemolysis of cells subjected to maximum centrifugation. Fig. 3 again shows the greater susceptibility to hemolysis induced by the alteration in the physicochemical properties of cellular suspensions produced by excessive centrifugation. Here, varying amounts of amboceptor are added to a constant volume of sheep-cell suspension and complement with the upper row of tubes

again representing cell suspensions prepared by the new technique and the lower row representing those suspensions prepared by the method of maximum centrifugation.

It is of interest to note that the daily complement and amboceptor titrations performed with cell suspensions made up in such a manner are remarkably uniform in view of the fact that the daily lots of sheep cells were from different animals. A great deviation, however, was observed in daily titrations using cells subjected to maximum centrifugation. Thought to be of lesser significance was the greater sensitivity to hypotonic saline of cells packed to minimum volume. The range of complete hemolysis in the samples of cells prepared in the new manner was lacking in the uniformity experienced in the amboceptor and complement titrations, but it was less than the equivalent range of hemolysis of the cells centrifuged to minimum volume. There was apparently little relation between the sensitivity of cells to hypotonic saline and their hemolytic sensitivity demonstrated by amboceptor and complement titrations.

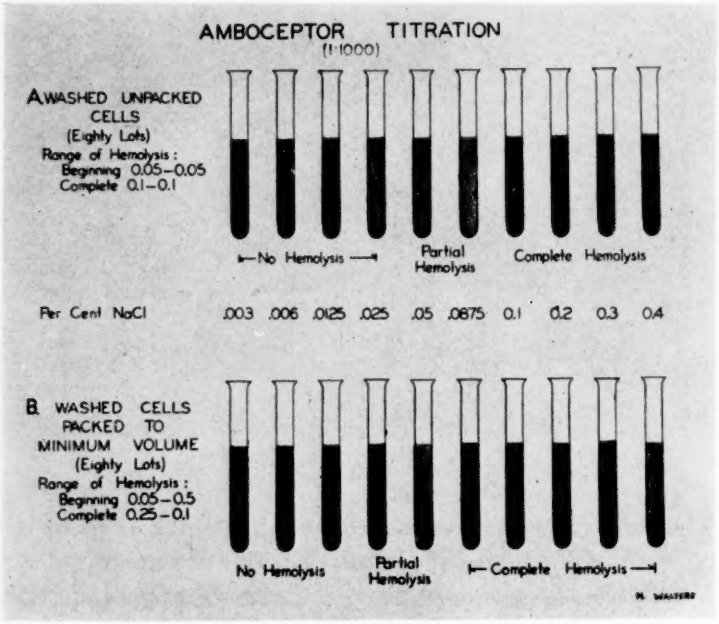

Fig. 3.

SUMMARY

1. The necessity of standardization of reagents for complement fixation reactions is stressed.

2. Of the five variables involved in such a reaction, the standardization of three, the antigen, the complement, and the hemolytic amboceptor, is dependent on a fourth variable, the 2 per cent sheep-cell suspension. The test proper, a titration of the fifth variable, the human serum, also employs the sheep-cell suspension as an indicator.

TABLE I

MEAN	PROBABILITY	EXPLANATION
Erythrocyte Fragility		
Beginning Hemolysis:		
A. 0.5800	0.05222800	Probability of 4 or more alike by chance
B. 0.5957	0.36269200	Probability of 3 or more alike by chance
Complete Hemolysis:		
A. 0.5250	0.05222800	Probability of 4 or more alike by chance
B. 0.5325	0.98185600	Probability of 2 or more alike by chance
Amboceptor Titration		
Beginning Hemolysis:		
A. 5.0000	0.00000010	Probability of 8 or more alike by chance
B. 5.0000	0.00000010	Probability of 8 or more alike by chance
Complete Hemolysis:		
A. 10.0000	0.00000010	Probability of 8 or more alike by chance
B. 8.7500	0.52228800	Probability of 4 or more alike by chance
Complement Titration		
Beginning Hemolysis:		
A. 16.2500	0.00431650	Probability of 5 or more alike by chance
B. 5.0000	0.00000010	Probability of 8 or more alike by chance
Complete Hemolysis:		
A. 30.0000	0.00000010	Probability of 8 or more alike by chance
B. 16.2500	0.00431650	Probability of 5 or more alike by chance

This table demonstrates the statistical significance of comparing eighty lots of 2 per cent sheep-cell suspensions prepared according to the new technique described in the text (A) with eighty lots of 2 per cent sheep-cell suspensions prepared by maximum centrifugation (B).

3. Preparation of such a suspension from cells subjected to maximum centrifugation frequently results in one of two undesirable ends: (a) actual disruption of cells resulting in visible hemolysis; and (b) alteration of the physicochemical qualities of the cells to such an extent as to render the cells more susceptible to hemolysis in the presence of amboceptor and complement as shown by: (1) altered erythrocyte fragility curves, and (2) altered hemolytic amboceptor titration.

4. If the cellular suspension is not prepared in such a manner, the suspension is often of a magnitude less than the prescribed 2 per cent. Furthermore, there will be a variation among such suspensions.

5. A technique is described of preparing true 2 per cent suspensions which compare favorably when subjected to turbidimetric spectrophotometric measurements and which does not involve subjecting the cells to be used in the complement fixation test to maximum centrifugation with its concomitant deleterious effects to the cells. The statistical significance of the data is shown.

The authors wish to emphasize their debt to Miss Martha Bickel of the Department of Clinical Pathology of the Cleveland Clinic Foundation for invaluable technical assistance.

The authors also acknowledge their indebtedness to Mrs. Lydia E. Kinzer of the Department of Mathematics of the Ohio State University for testing the statistical significance of the titrations and erythrocyte fragility studies.

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IMMUNITY RESPONSE IN MUMPS WITH COMPLICATIONS

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IN A STUDY of the antibody and skin test responses of mumps patients at various time intervals after the onset of their clinical disease, it has become possible to compare the results in cases with and without complications.

MATERIALS AND METHODS

Virus.—Mumps virus originally adapted to the chick embryo in this laboratory[1] was used in the complement fixation, virus neutralization, and skin tests. Separate substrains were carried in chick embryo by yolk sac and by allantoic sac passage.

Skin Tests.—Early in this study both yolk sac and allantoic fluid mumps virus substrains were used to prepare skin test antigens. Through most of the study, only undiluted allantoic fluid antigen has been employed, comparable normal chick embryo materials being used as control antigens. Skin test antigens were prepared by heating the infected 10 per cent yolk sac suspension supernate or the undiluted allantoic fluid to 65° C. for ten minutes and storing at −70° C. until used. Skin tests were read in twenty-four to thirty hours after 0.1 c.c. had been given intradermally on the forearm. A positive skin test reaction was based on a definitely outlined area of erythema at least 10 mm. in diameter when the reaction to the control material was less than 5 mm. A questionably positive reaction was characterized by a very light erythema occurring at the site of inoculation, the outline of which was so indefinite that exact measurements could not be made.

Complement Fixation.—The technique of this test was as previously described.[1] Infected yolk sac antigens were used routinely. All human sera were inactivated at 60° C. for twenty minutes before the test.

Virus Neutralization.—The technique for this procedure has been previously described.[2] It involves the inoculation of chick embryos with virus-serum dilution mixtures after incubation, the harvest of chick embryo materials and their subsequent testing for evidence of virus multiplication by complement fixation against a known positive mumps serum. For the neutralization done in this present study, human serums were not inactivated before use in the test.

Procedure.—A diagnosis of mumps was made entirely on the basis of painful swelling of one or more of the salivary glands. Considered as complications were orchitis, meningoencephalitis, pancreatitis, and late spread to salivary glands other than that originally involved. A total of ninety-seven men and nine women patients were skin tested and bled at various intervals after the onset of their clinical mumps. Due to the relatively short hospital stay of these patients in only a few individuals[7] were more than one skin test or bleeding done. Of forty-six complicated cases of mumps forty-two had orchitis, one meningoencephalitis, one acute pancreatitis, and two late spread to other salivary glands.

RESULTS

In Table I are listed the skin test reactions of fifty-six uncomplicated cases of mumps according to the day of disease when tested. Even as early as the first day of any clinical manifestations of mumps, the majority of the reactions were positive. In the first five days, thirty-two of thirty-eight, or 84.2 per cent,

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were definitely positive. However, in twenty-four cases with complications tested in the first five days of disease only three, or 12.5 per cent, were definitely positive. Even in the second five days of disease eleven of fourteen complicated cases were either negative or questionable skin test reactions. After the tenth day of mumps practically all cases of the small number tested were skin-test positive. Of all uncomplicated cases tested at various intervals, 81 per cent were positive while only 22 per cent of complicated cases reacted.

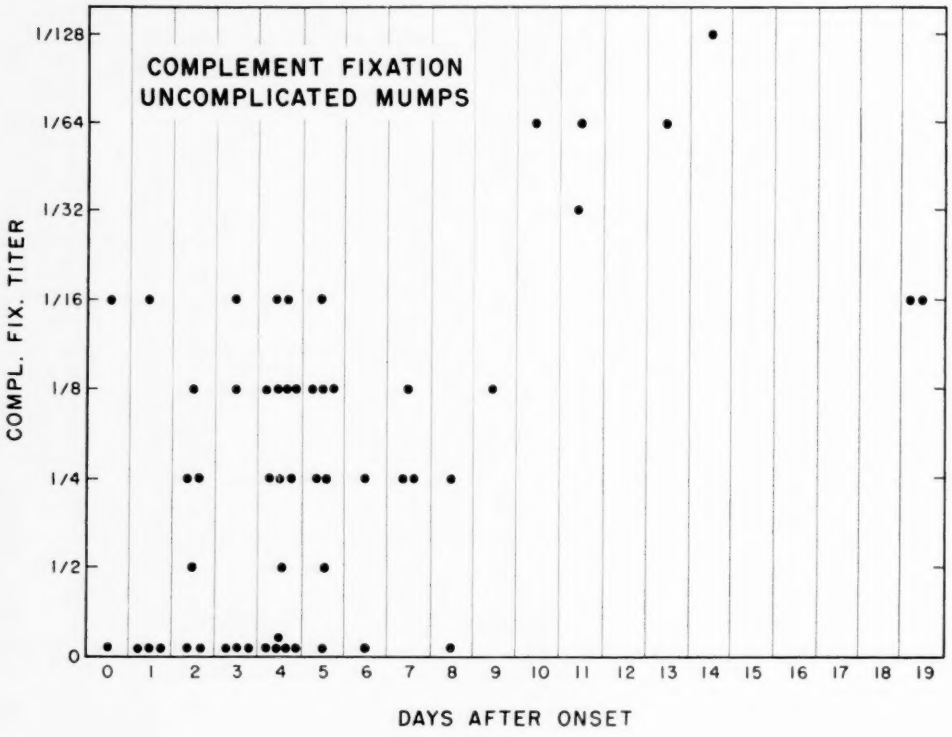

Fig. 1.

Sera of fifty-four uncomplicated and forty-eight complicated cases of mumps were tested by complement fixation. The distribution of titers in relation to stage of the clinical disease is shown in Figs. 1 and 2. It can be seen that substantial amounts of complement-fixing antibody were present in the uncomplicated cases starting at the second day of illness, and indeed were present in two cases prior to that. In the complicated cases, there is a lag of two to three days before comparable antibody levels are reached. From the fifth day to about the fourteenth day the titers of serums from complicated cases rise rapidly to equal those from uncomplicated cases, and in both groups are dropping by the end of the third week.

Neutralization titers were obtained on fifty-two sera from uncomplicated and forty-six from complicated cases of mumps. The relation of titers to stage of disease in the two types of cases here was the same as with the complement

TABLE I. SKIN TEST REACTIONS IN UNCOMPLICATED AND COMPLICATED CASES OF MUMPS

SKIN TEST REACTION	UNCOMPLICATED MUMPS—DAYS OF DISEASE				COMPLICATED MUMPS—DAYS OF DISEASE			
	TOTAL	1 TO 5 DAYS	6 TO 10 DAYS	OVER 10 DAYS	TOTAL	1 TO 5 DAYS	6 TO 10 DAYS	OVER 10 DAYS
Positive	45	32	8	5	10	3	3	4
Questionably positive	2	2	0	0	11	7	4	0
Negative	6	4	1	1	21	14	7	0
Control and antigen reaction equal	3	2	1	0	4	3	0	1
Total	56	40	10	6	46	27	14	5

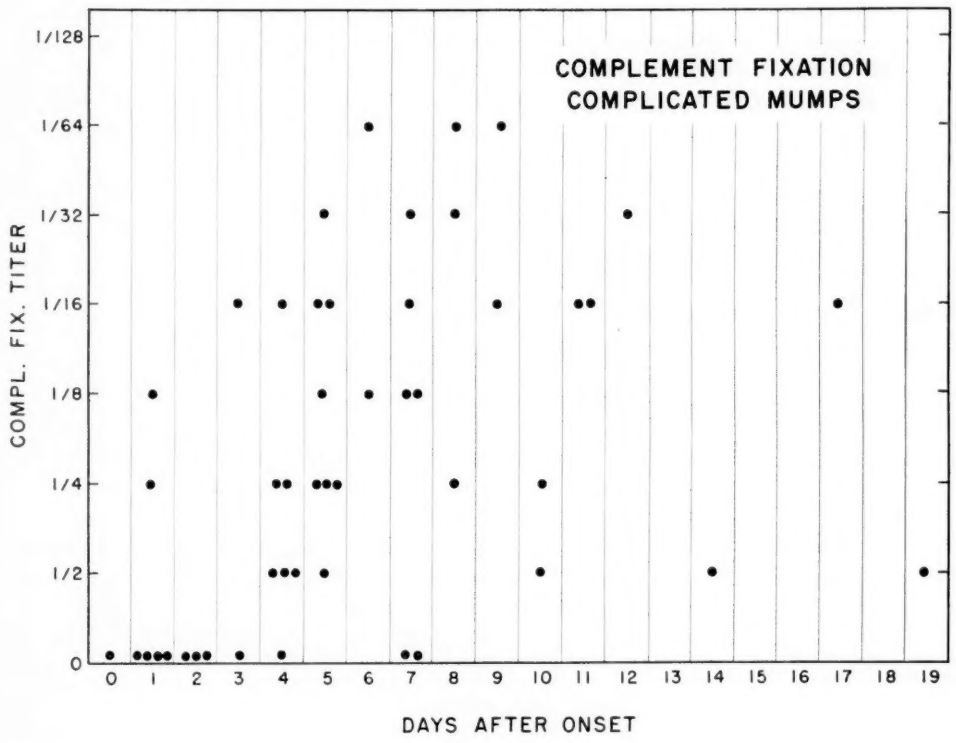

Fig. 2.

TABLE II. RELATION OF COMPLEMENT-FIXING ANTIBODY TITERS TO SKIN REACTIONS DURING FIRST FIVE DAYS OF MUMPS

SKIN TEST REACTIONS	UNCOMPLICATED MUMPS—SERUM TITERS			COMPLICATED MUMPS—SERUM TITERS		
	NEGATIVE	½ TO ⅛	1/16 OR OVER	NEGATIVE	½ TO ⅛	1/16 OR OVER
Negative or questionable	3	3	0	8	8	4
Positive	10	15	7	1	4	1
Total	13	18	7	9	12	5

TABLE III. RELATION OF VIRUS NEUTRALIZING ANTIBODY TITERS TO SKIN REACTIONS DURING FIRST 5 DAYS OF MUMPS

SKIN TEST REACTION	UNCOMPLICATED MUMPS			COMPLICATED MUMPS		
	SERUM TITER			SERUM TITER		
	NEGATIVE	UNDILUTED TO 1/4	1/10 OR OVER	NEGATIVE	UNDILUTED TO 1/4	1/10 OR OVER
Negative or questionable	3	3	0	7	8	1
Positive	14	17	0	4	2	0
Total	17	20	0	11	10	1

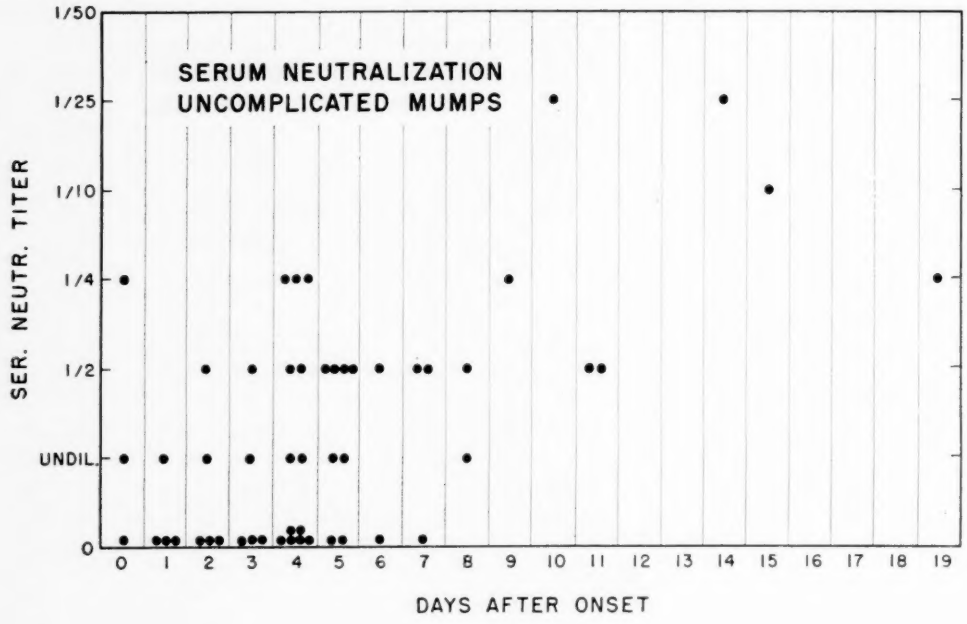

Fig. 3.

fixation tests. Neutralizing type of antibody tended to be present more often and in higher amounts in the first five days of uncomplicated mumps than in the same stage of complicated cases (Figs. 3 and 4).

The relation of antibody titers to skin test reactions during the first five days of mumps is not clear cut, but in general the negative skin reactors tend to have lower antibody titers as seen in Tables II and III.

DISCUSSION AND SUMMARY

Using chick embryo propagated mumps virus for skin testing; it was surprising to find that many cases of mumps were already skin test positive in the first few days of their clinical disease. This was at variance with the results obtained by Enders and associates[3] when using monkey parotid gland mumps virus as skin test antigens. They found that skin tests in the first four days of parotitis were negative in 95 per cent of cases. However, not all mumps cases in the present study were skin-test positive early in their clinical course, and subsequent occurrence of clinical complication appeared to be the differentiating factor.

Of thirty-eight patients who subsequently had no clinical complications, 84 per cent had positive skin tests in the first five days of their illness, while of twenty-four who subsequently did develop complications only 12 per cent were positive.

It was also found that both complement-fixing and virus neutralization antibody titers were often elevated early in the course of clinical mumps. A comparison between complicated and uncomplicated cases showed that antibody response was slower in developing and in the early stages quantitatively less in those cases subsequently developing complications.

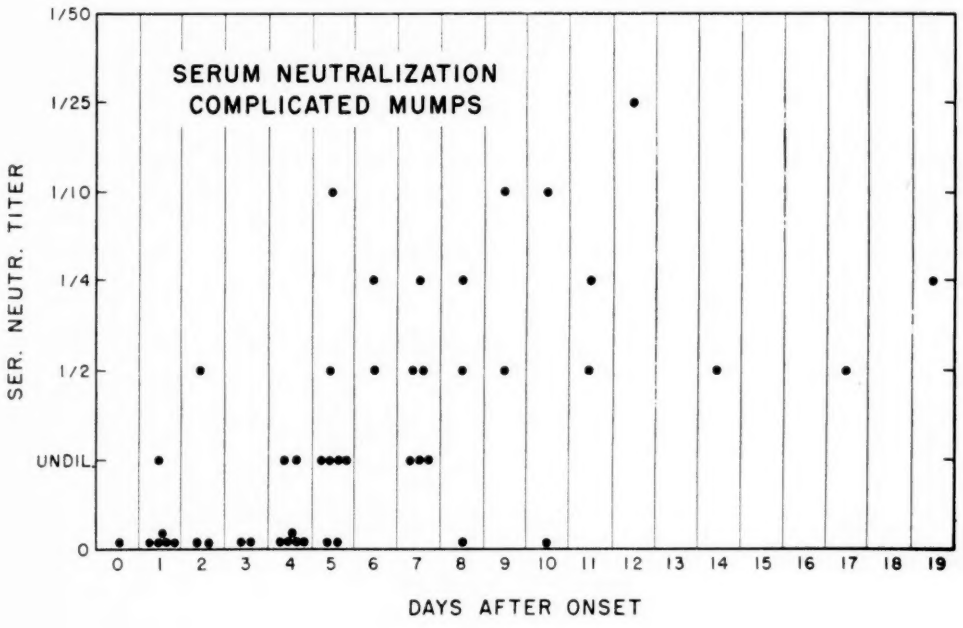

Fig. 4.

It is not too surprising that antibodies should be present at the onset of mumps in view of the relative incubation period. Evidence is now quite definite that the complications of orchitis and meningoencephalitis in mumps are actually due to invasion of those organs with mumps virus.[4, 5] It is assumed that this wide distribution of the virus is by way of the blood stream although attempts to isolate mumps virus from the blood of patients have been successful in only one reported instance.[6] Clinical studies have shown that mumps immune serum gamma globulin administered in the early stage of clinical mumps in adult males reduces the subsequent incidence of orchitis.[7] Nonspecific therapy by the administration of diethylstilbestrol has also been reported as promising in the prevention of mumps orchitis,[8] but others have not confirmed this.[9]

This evidence on the possible role of immunity as manifested by skin test reaction and antibody titer in determining the occurrence of complications in mumps is consistent with the hypothesis that the use of hyperimmune mumps serum given early in the course of the disease might be of value in preventing

subsequent complications. The results here reported in the correlation between early skin test results and the subsequent occurrence of complications offers a limited method of determining in advance those cases of mumps in adult males who are likely to develop orchitis and therefore give a means of deciding which should receive preventive therapy.

The cooperation of the members of the contagious disease service of the U. S. Naval Medical Center, Bethesda, Md., was invaluable in making clinical cases of mumps available for this study.

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LABORATORY METHODS

RAPID TURBIDIMETRIC ASSAY OF CHOLESTEROLS

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CHOLESTEROL determinations based on four different principles have been recommended. The tediousness of the gravimetric method, the inaccuracy of the titrimetric method, and the unreliability of the several colorimetric techniques are well known. Only the combined digitonin precipitation and colorimetric determination of Schoenheimer and Sperry[1] gives reproducible results.

The Schoenheimer-Sperry method and its modification have disadvantages: (a) they are time consuming, involving a multitude of manipulations, (b) they are lengthy, requiring overnight standing, (c) they are oversensitive due to color change with even slight variations of temperature and of the length of the incubation, and (d) the color of the Liebermann-Burchard reaction is not stable.

A turbidimetric method is hereby presented. It renders results comparable to the Schoenheimer-Sperry technique, while it is free of the disadvantages of colorimetric methods. Generally, our method follows that of Schoenheimer-Sperry; it differs inasmuch as digitonin precipitation constitutes the final phase of our technique. Using the turbidimetric method, a series of fifty blood samples can be processed during a working day. Thus, the method can be employed as a screening test.

Reagents.—

(a) Acetone-alcohol mixture. Equal parts of acetone (c.p.) and 95 per cent ethyl alcohol.

(b) Potassium hydroxide solution. Dissolve 10 Gm. of potassium hydroxide (c.p.) in 20 ml. of distilled water.

(c) Phenolphthalein, 1 per cent solution. Dissolve 1 Gm. of phenolphthalein in 95 per cent ethyl alcohol and dilute to 100 ml.

(d) Acetic acid, 10 per cent. Dilute 10 ml. of glacial acetic acid (c.p.) to 100 ml. with distilled water.

(e) Digitonin solution, 0.5 per cent. Dissolve 500 mg. of digitonin (c.p.) in 100 ml. of 50 per cent ethyl alcohol at 60° C.

(f) Stock cholesterol solution. Dissolve exactly 100 mg. of cholesterol (Pfanstiehl, c.p.) in 100 ml. of acetone-alcohol mixture. (Use volumetric flask.)

(g) Working standard, I. Dilute 1 ml. of the stock cholesterol solution to 25 ml. with the acetone-alcohol mixture.

(h) Working standard, II. Dilute 2 ml. of the stock cholesterol solution to 25 ml. with the acetone-alcohol mixture.

Special Equipment.—

(a) Nephelometer, Coleman. (Other instruments could be used.)

(b) Test tubes, calibrated; to fit the optical instrument.

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(c) Graduates, glass stoppered, Pyrex brand, 25 ml. capacity.

(d) Stirring rods, glass, about 13 cm. long and 3 mm. thick, with bulbar tips.

(e) Preserving jar containing fine sand to a height of about 5 cm.

Extraction of Serum (or other material).—

(a) Place 1 ml.* of serum into a graduate.

(b) Rapidly add 10 ml. of acetone-alcohol mixture; stopper and shake.

(c) Break up protein clumps with glass rod.

(d) Place graduate into boiling water bath; bring contents to a boil.

(e) Allow to cool to room temperature.

(f) Add acetone-alcohol mixture to make a volume of 25 ml.

(g) Filter the extract.

Determination of Total Cholesterol.—

(a) Transfer 3 ml. of filtrate to calibrated (nephelometer) tube.

(b) Add 0.1 ml. of potassium hydroxide solution.

(c) Stir vigorously with glass rod until no more droplets of the alkali solution can be seen at the bottom of the tube. Do not remove glass rod!

(d) Place tube into preserving jar with sand preheated to 45° C.; cover the jar and place for thirty minutes into incubator at 37° C.

(e) Transfer tube to rack and allow to cool to room temperature.

(f) Add 3 ml. of acetone-alcohol mixture.

(g) Add 2 drops of phenolphthalein indicator (solution turns pink).

(h) Add, drop by drop, acetic acid until complete decolorization (neutralization), and add 1 drop of acetic acid in excess.

(i) Add 3 ml. of digitonin solution and stir vigorously.

(j) Allow to stand for one hour at room temperature.

(k) Stir till suspension is uniformly turbid and free of particles; remove glass rod.

(l) Transfer tube to nephelometer and read promptly.

Determination of Free Cholesterol.—

(a) Transfer 3 ml. of filtrate to calibrated (nephelometer) tube.

(b) Add 3 ml. of acetone-alcohol mixture.

(c) Add 1 drop of acetic acid.

(d) Add 3 ml. of digitonin solution and stir vigorously.

(e) Allow to stand for one hour at room temperature.

(f) Stir till suspension is uniformly turbid and free of particles; remove glass rod.

(g) Transfer tube to nephelometer and read promptly.

Preparation of Standards.—

Three milliliters of each of the two working standards (I and II) are treated the same way as the extracts of unknown samples.

Notes.—

(a) Preparation of multiple standards is imperative because reading of standards for free cholesterol are higher than readings of standards for total cholesterol due to more rapid precipitation of the free cholesterol.

(b) To avoid differences in evaporation, aliquots for determination of total and free cholesterol and standards are pipetted at the same time.

(c) As long as extracts and standards are processed simultaneously, readings will be correct regardless of whether made exactly one hour after the addition of digitonin solution or later.

Calculations.—

$$\frac{\text{reading of unknown}}{\text{reading of standard I}} \times 100 = \text{mg. cholesterol per 100 ml.}$$

$$\frac{\text{reading of unknown}}{\text{reading of standard II}} \times 200 = \text{mg. cholesterol per 100 ml.}$$

*Use 0.5 ml. if serum icteric, if a high blood cholesterol is expected (over 300 mg. per 100 ml.), and for repeat tests.

EXPERIMENT

Series of healthy, walking blood donors and of patients of all age groups and hospitalized for various reasons were studied simultaneously by the turbidimetric method and by the (colorimetric) method of Schoenheimer and Sperry. The total number of blood samples analyzed to date is 350. In Table I, twenty representative results are presented. The first ten subjects had nor-

TABLE I. COMPARISON OF BLOOD CHOLESTEROL VALUES ARRIVED AT BY THE TURBIDIMETRIC AND THE COLORIMETRIC METHOD (TWENTY EXAMPLES)

SUBJECT NO.	TURBIDIMETRIC METHOD				COLORIMETRIC METHOD			
	TOTAL	FREE	ESTERS	RATIO	TOTAL	FREE	ESTERS	RATIO
13	126	52	74	41.3	124	35	89	28.2
24	160	53	107	33.2	165	38	127	23.0
36	150	57	93	38.0	156	38	118	24.4
43	147	48	99	32.6	152	35	117	23.0
45	195	80	115	41.0	188	55	133	29.2
75	139	65	74	46.8	146	44	102	30.1
77	183	68	115	37.2	189	55	134	29.1
79	183	63	120	34.4	186	47	139	25.2
80	139	48	91	34.5	144	35	109	24.3
89	120	43	77	35.8	120	32	88	26.7
152	258	110	148	42.7	268	72	196	26.8
171	288	105	183	36.5	285	88	197	30.8
180	316	143	173	45.4	315	88	227	28.0
181	299	126	173	42.1	295	73	222	24.7
185	344	166	178	48.3	342	94	248	27.5
161	195	170	25	87.3	192	134	68	70.0
163	226	208	18	92.0	218	170	48	78.0
164	183	116	67	63.5	185	94	91	50.8
165	131	70	61	53.5	136	67	69	49.4
170	188	100	88	53.4	182	76	116	41.8

mal blood cholesterols. The next five persons had hypercholesterolemia. In the last five persons, the ratio of free to total blood cholesterol was disturbed. In the table, all cholesterol values are expressed in mg. per 100 ml. of blood; the ratio indicates the amount of free cholesterol in per cent of total cholesterol.

Cholesterol (e.p., Pfanstiehl) and/or cholesterol acetate (special recrystallized, No. 379, Reagent Chemical, Inc.) were added to pooled human serum. The amount of cholesterol and of cholesterol ester added were 10, 20, 50, and 100 mg. per 100 ml., respectively. The results of recoveries were excellent with either method.

DISCUSSION

The values for total blood cholesterol arrived at by the two methods compare well. The differences do not exceed the limits of technical errors inherent to laboratory methods. They hardly exceed the variations of repeat determinations by the same method. Such differences do not have clinical significance.

There is a distinct difference in free blood cholesterol as determined by the two methods. Turbidimetry yields always higher values than colorimetry. This is due to the fact that in the Schoenheimer-Sperry method the "impurities" ("cholesteroids"), that is, noncholesterol substances precipitated by digitonin, are removed by repeated washing of the precipitate. (Unless the washing is

done by an expert technician there is danger of erroneously low results due to loss of precipitate.) In the turbidimetric method, washing is not feasible because the cholesterol digitonide cannot be resuspended.

Where the ratio of free to total cholesterol is completely reversed, the nephelometric reading for free cholesterol approaches the reading for total cholesterol. In such an instance, turbidimetric determination of the free cholesterol is impossible.

Schoenheimer and Sperry considered 150 to 250 mg. per 100 .. the normal value for total blood cholesterol, and they held the free cholesterol value of 25 to 30 per cent of the total cholesterol normal. Using their technique for the assay of cholesterols in the blood of walking blood donors, we arrived at normals ranging from 120 to 200 mg. per 100 ml. for total cholesterol, with from 23 to 30 per cent of free cholesterol.

There are various reasons for the marked differences in the "normal values" reported in various texts. Results depend on the procedure used. Some investigators base their "normals" on the study of healthy college students. Others compare the results in middle-aged persons afflicted with diseases in which blood cholesterols are frequently altered with persons of the same age but without such disease and use the cholesterol values in the second group as "normals."

We concluded that the total blood cholesterol level normally ranges between 120 and 200 mg. per 100 ml., regardless whether our turbidimetric method or Schoenheimer-Sperry's method is employed. The free cholesterol normally equals 23 to 30 per cent of the total blood cholesterol, with the Schoenheimer-Sperry technique, and 28 to 48 per cent, with the turbidimetric method.

The broad range of free cholesterol loses its significance if one utilizes its upper limit to differentiate between normal and abnormal. A ratio of free to total cholesterol in excess of 30 per cent should be held abnormal if one uses the Schoenheimer-Sperry method. A ratio higher than 48 per cent should be considered excessive if the turbidimetric method is employed.

SUMMARY

A speedy and simple turbidimetric method for the assay of cholesterols is presented.

This turbidimetric method is compared with Schoenheimer-Sperry's colorimetric method.

The normal total blood cholesterol equals 120 to 200 mg. per 100 ml., regardless whether the turbidimetric or the colorimetric method is employed.

The ratio of free cholesterol to total cholesterol is abnormal if it exceeds 30 per cent by the colorimetric method or if it exceeds 48 per cent by the turbidimetric method.

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A MICROMODIFICATION OF ARCHIBALD'S COLORIMETRIC DETERMINATION OF UREA

A Rapid Production of a Linear, Reproducible Color by Heating a Strongly Acid Solution of Urea and α-Iso-Nitrosopropiophenone at Increased Pressure

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ARCHIBALD developed a urea determination method using α-iso-Nitrosopropiophenone as the colorimetric reagent.[1] Halvorson and Schultze adopted the method to investigate blood urea concentrations among newborn rats.[2] These workers found that the absorbance (optical density) of the colored compound was directly proportional to the concentration of urea in a limited range. The method is based upon the formation of a colored compound when strongly acid solutions of urea and α-iso-Nitrosopropiophenone are heated together. This paper presents an accurate and a rapid micromodification which simplifies Archibald's procedure, and increases the useful range as well. There is a timesaving factor since the reaction is allowed to take place at a higher temperature in order that the method can be completed quickly. In addition to these, conditions have been found where the system conforms to Beer's law, so that calculations can be made on the assumption that the absorbances (optical densities) of unknowns and standards are proportional to their concentrations. Furthermore, the per cent relative analysis error per 1 per cent absolute photometric error[3] has been diminished.

APPARATUS

An autoclave† or a large pressure cooker.

Spectrophotometer‡ or photoelectric colorimeter.

Pipettes: Two types of pipettes are used for this method—(1) syringe pipettes[4, 5] modified for semiautomatic delivery,§ and (2) and overflow type of micropipette of Harkins[6] modified by the writer (Fig. 1). The syringe pipettes are extremely convenient to handle and are extraordinarily accurate—the accuracy of a milliliter syringe is about 0.01 per cent.[7] Furthermore, when the same volume of a reagent is to be added to a large number of samples, these pipettes will save much time. On the other hand, they are not suited for transferring many different samples, because the dead space requires a thorough cleaning between each sample. When transferring samples, an overflow type of micropipette, described below, is more convenient.

The accuracy of 0.1 ml. overflow micropipette is 0.05 per cent when manipulated with reasonable care. The overflow self-adjusting micropipette with interchangeable ground glass connection standard taper $^{7}/_{25}$, has been found to be the most practical form.‖ This facilitates the connecting of the pipette to the rubber tubing, which in turn has the complement of the standard taper joint.

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†An electric autoclave Model LV of The Pelton and Crane Company, 622 Harper Avenue, Detroit 2, Mich., is used in this laboratory.

‡The Beckman Model B spectrophotometer is used in this laboratory.

§The syringe pipette frame may be obtained from Mr. Herman Ruf, Northern Tool and Instrument Co., 5023 192nd Street, Flushing, Long Island, N. Y.

‖Obtainable from A. S. Aloe Co., 1831 Olive Street, St. Louis 3, Mo.

In principle and use, the overflow micropipettes are similar to Lang-Levy constriction pipettes.[8, 9] They are filled and emptied by the use of a small rubber tubing, with standard taper 7/25 female ground glass joint at one end, similar to that used with blood-diluting pipettes. The tip of the pipette is not dipped more than 2 mm. into the fluid, and, by sucking, the liquid is pulled to the upper capillary. With a little practice no fluid will overflow; this facilitates the manipulation of the overflow, self-adjusting micropipette. During delivery, the tip of the pipette is kept in tight contact with the wall of the test tube into which the sample is being delivered. It is important for the tip of the pipette always to touch the test tube wall during delivery since, if the tip is free in the air, much of the sample will adhere to the outside of the pipette.

The overflow micropipettes are relatively easily washed by attaching to an aspirator through a rubber tubing in the usual manner.

Fig. 1.—An overflow micropipette with interchangeable ground glass joint standard taper 7/25.

REAGENTS

A. *For the precipitation of blood proteins.*[10]—

Stock Acid Cadmium Sulfate: In a 1,000 ml. volumetric flask place 13.9 Gm. of crystalline cadmium sulfate ($3CdSO_4.8H_2O$) or 10.59 Gm. anhydrous cadmium sulfate ($CdSO_4$) and 63.5 ml. of exactly 1.0N sulfuric acid. Add approximately 900 ml. of distilled water and shake until dissolved; dilute to volume with distilled water.

Sulfuric Acid: Volumetric 1.0N.

Sodium Hydroxide: 1.1N.

0.055N Sodium Hydroxide: Prepared from 1.1N NaOH.

Dilute Acid Cadmium Sulfate: Take 100 ml. of stock acid cadmium sulfate and make up to 250 ml. mark with distilled water.

B. *For color development.*—

Sulfuric-Phosphoric Acid Mixture: One volume of concentrated sulfuric acid, three volumes of syrupy phosphoric acid, and 1 volume of water are mixed together.

α-iso-Nitrosopropiophenone:* Prepare 4 per cent solution in 95 per cent ethanol.

Urea Standard: Weigh 34.4 mg. of desiccator dried urea crystals and transfer into 100 ml. volumetric flask and make up to the mark with distilled water. This standard will contain 160 mg. of urea N per 100 ml. Keep refrigerated when not in use. Prepare fresh monthly.

PROCEDURE

A. *Precipitation of protein.*—

Add 0.1 ml. of blood (or diluted urine) into 2.0 ml.* of the dilute acid cadmium sulfate solution in a 12 ml. Pyrex centrifuge tube. Let stand for one minute. Add 2 ml.* of 0.055N sodium hydroxide. Mix well with paraffined wooden applicator. Then centrifuge for five minutes at 2,500 r.p.m.

B. *Color development.*—

Add 2.0 ml.† of distilled water into cuvettes (20 × 170 mm. "S" tubes or 19 × 105 mm. coleman round cuvettes are recommended). Add 1.0 ml. of centrifugate with volumetric pipette. Add 0.5 ml. of 4 per cent alcoholic solution of α-iso-Nitrosopropiophenone (using 1 c.c. syringe pipette adjusted to deliver this volume). Finally add 4.0 ml. of sulfuric-phosphoric acid mixture (using 10 c.c. syringe pipette adjusted to deliver this amount). Shake very well to disperse any crystals of colorimetric reagent which may have floated to the surface of the solution. Finally autoclave the tubes for exactly twenty minutes at 20 pounds pressure (or thirty minutes at 15 pounds pressure) in a preheated apparatus. At the end of the specified period, release the pressure rapidly, and remove the tubes from the autoclave as soon as the atmospheric pressure is reached. Then place them into a cool water bath (about 10° to 15° C.) for five minutes.‡

Make reagent blank by treating 1 ml. of distilled water in the same manner as 1 ml. of centrifugate, and set the spectophotometer (or a photoelectric colorimeter) at 0 absorbance (100 per cent transmission) with light at 545 mμ§ (use 540 green filter in photoelectric colorimeter) wave length.

Then record absorbances (or optical densities).

CALIBRATION

Dilute the urea standard, which contains 160 mg. of urea N per 100 ml., to make 80.0, 40.0, 20.0, 10.0 and 5.0 mg. urea N per 100 ml.

Add 0.1 ml. of standards with an overflow type of micropipette into 12 ml. Pyrex centrifuge tubes containing 2.0 ml., of dilute acid cadmium sulfate in the ascending order. The reagent blank and the standards are given the same treatment as blood or urine centrifugates.

Under the conditions specified, a straight line relationship was found between the concentrations and the absorbances (optical densities).

For urea, multiply urea N value by 2.14.

For the determination of urine urea N, dilutions of 1:5, 1:10, 1:20, or 1:40 will give a readable color (use those values which will fall within the range of urea concentrations

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*Obtain from the Eastman Kodak Company or from Anachemia, Lts., Montreal, Canada, and 70 East 45th Street, New York 17, N. Y.

†Use 5 c.c. syringe pipette adjusted to deliver this volume. Wash with distilled water three times before using it for the next solution. All syringe pipettes must be cleaned and then filled with distilled water when not in use.

‡If you have fluorescent lights, cover them with black cloth until the spectrophotometric determinations are done.

§An experiment with the Beckman DU spectrophotometer has shown that the maximum absorption takes place at 545 mu.

recommended for the method, i.e., 8 to 80 mg. of urea N per 100 ml., as indicated in Fig. 4, curve 2).

CALCULATION

Plot standard curve milligrams of urea nitrogen as abscissas and absorbances (optical densities) as ordinates. Then calculate unknowns graphically (or plot concentration against corresponding per cent Transmittance [T] on a semilogarithmic paper). Or conversion factor (K) calculated: milligrams urea N per 100 mg. $= K_1 (2 - \log\%T)$; $\%T =$ per cent transmission.

EXPERIMENTAL

In his studies of urea derivatives with α-iso-Nitrosopropiophenone ($C_6H_5.CO.CNOH.CH_3$) at 100° C., Archibald found a number of interesting reactions.[1]

In an attempt to simplify the procedure, minimize working time, and to insure the accuracy of the modifications just described, the following conditions were investigated in this laboratory:

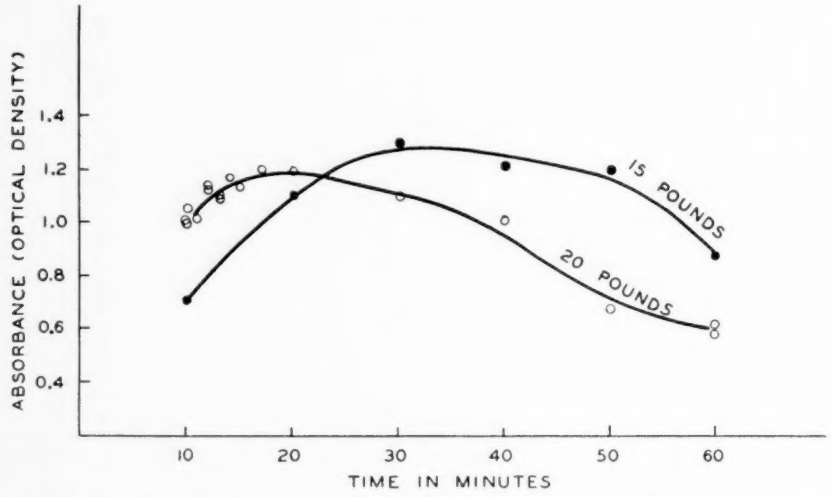

Fig. 2.—The effect of variation of autoclaving time at 15 pounds and 20 pounds pressures.

The Effect of Variation of Autoclaving Time.—The results of the experiment are shown in Fig. 2. The optimum autoclaving time for the procedure described were found to be twenty and thirty minutes for 20 and 15 pounds pressures, respectively. A similar investigation using a large pressure cooker at 15 pounds pressure gave results essentially the same as that of the autoclave study.*

The Effect of Variation of the Amount of α-iso-Nitrosopropiophenone Used.—To 1.0 ml. of Fujita-Iwatake[10] filtrate 2.0 ml. of distilled water and 4.0 ml. of sulfuric-phosphoric acid mixture were added. Then the varying amounts of 4 per cent alcoholic solution of α-iso-Nitrosopropiophenone, the colorimetric reagent, were added. The results are shown in Fig. 3. The maximum absorbances (optical densities) were found between 0.2 ml. to 0.8 ml., 0.5 ml. being the geometric mean of the plateau.

*In any case, prolonged heating will completely destroy the red color formed.

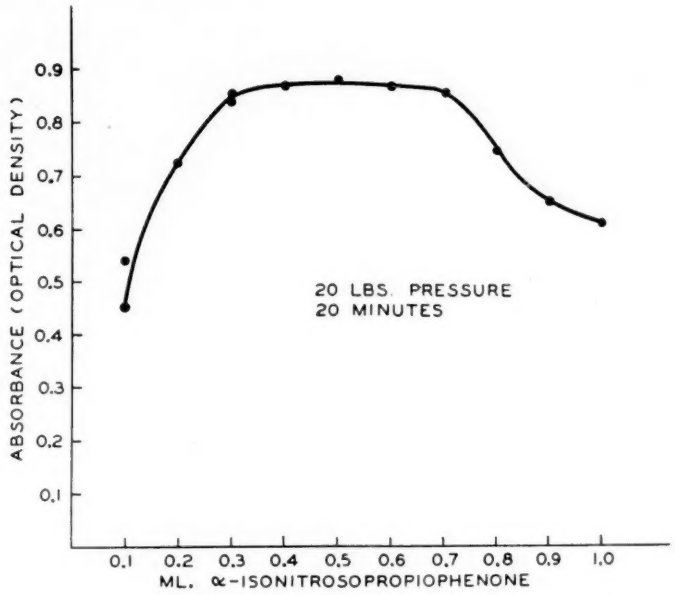

Fig. 3.—The effect of variation of the amount of α-iso-Nitrosopropiophenone used.

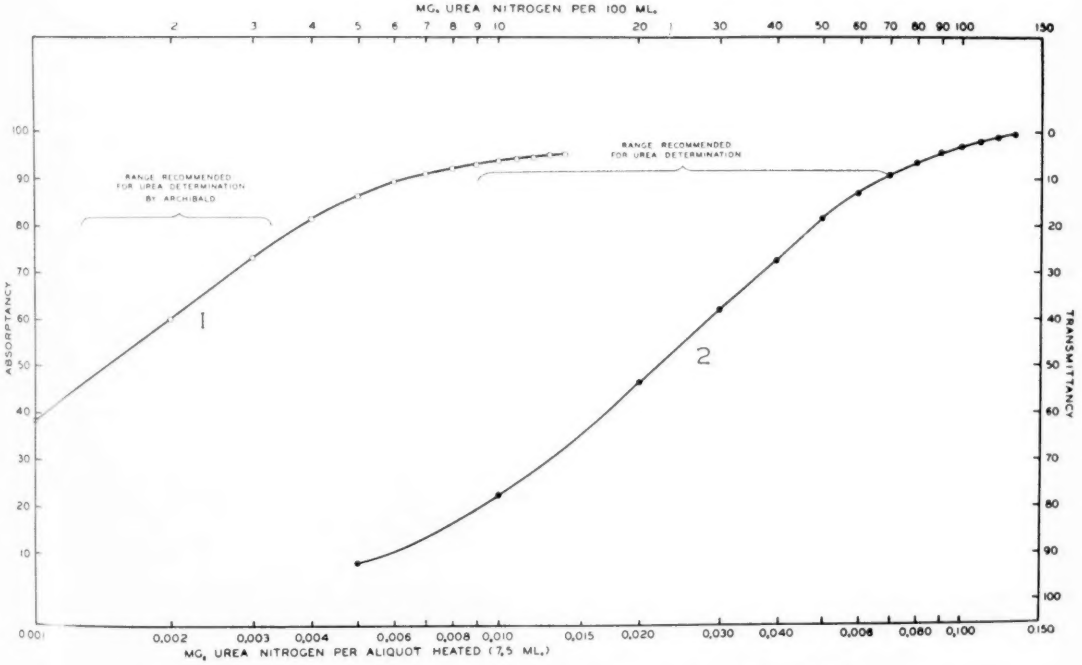
MG. UREA NITROGEN PER 100 ML.
2 3 4 5 6 7 8 9 10 20 30 40 50 60 70 80 90 100 150
RANGE RECOMMENDED FOR UREA DETERMINATION BY ARCHIBALD
RANGE RECOMMENDED FOR UREA DETERMINATION
1
2
ABSORPTANCY
TRANSMITTANCY
0.001 0.002 0.003 0.004 0.006 0.008 0.010 0.015 0.020 0.030 0.040 0.060 0.080 0.100 0.150
MG. UREA NITROGEN PER ALIQUOT HEATED (7.5 ML.)

Fig. 4.—Standard curves for red color produced in colorimetric determination of urea. Curve 1 as given by Archibald. Curve 2 shows the increase in the range recommended for urea determination by the modification described.

In this laboratory it has been found that 0.5 ml. of 4 per cent alcoholic solution of α-iso-Nitrosopropiophenone is adequate under the conditions specified.

The Effect of Variation of the Ratio of H_2SO_4:H_3PO_4:H_2O.—The ratio of 1:3:1 was found to be the optimum for the procedure described.

Treatment of Blood and Urine with Urease in Order to Determine Quantitatively the Total Urea N.—To each of several 10.0 ml. aliquots of human blood was added 0.5 Gm. (Squibb) urease, and these were allowed to incubate for one hour at 25° C. Then 0.1 ml. aliquots were used to determine the amount of nonurea constituents detectable by the procedure described. (As Archibald has already found,[1] neither ammonia nor products ordinarily encountered in human blood filtrates interfere appreciably with the analysis.) No interference substances were found in the normal human blood used for the experiments.* However, there are known to be present in some human urine and blood substances other than urea, which react with the colorimetric reagent. Some of this material is believed to be allantoin.[1]

Essentially the same results were obtained with human diluted urine when aliquots treated with urease were processed to obtain the reagent blanks. This procedure automatically removes the absorbances (optical densities) due to nonurea substances.

Recovery of Added Urea.—The customary recovery experiments were performed with the whole blood for the procedure described. When 5.0, 10.0, 15.0, and 20.0 mg. urea N were added per 100 ml. of blood, the percentage recoveries were 100.0, 99.0, 99.5 and 99.0, respectively. These percentage recoveries indicate, of course, the over-all accuracy of the method.

Evaluation of Accuracy of the Modified Method.—As suggested by Ayres,[3] the data are plotted in the form of absorptancy against logarithm of concentration. This is shown in curve 2, Fig. 4. The optimum range can be defined by inspection of the curve, as it is the concentration range in which it has its steepest slope. The maximum attainable accuracy† is easily derived from the slope of the curve; the very flat portions of the curve at low and at high absorptancy emphasize the importance of selection of proper range if best accuracy is to be realized. For the proposed method the range of best accuracy is from 8 to 80 mg. of urea N per 100 ml. (0.008 to 0.08 mg. of urea N per aliquot autoclaved). From the curve 2, Fig. 4, it can be seen that there is a gain of the range over the method previously described[1, 11] from curve 1, Fig. 4.

*Thymol causes the development of turbidity with the colorimetric reagent[1] under the conditions specified in this paper.

†A method of reducing the photometric reading error is provided on the Beckman spectrophotometer in the form of a selector switch. By the use of this selector switch the transmittancy reading can be made to a precision as good as 0.02 per cent transmittancy, and can thus extend the range and actually increase the accuracy at transmittancies somewhat below 11 per cent.[3] As the Beckman Model B spectrophotometer which was used for this investigation, there are four positions on the dial which control the amount of amplification through selection of resistor values in the feedback circuit of the amplifier. Position 1 represents the lowest gain position, and consequently requires the widest slit width. With each change in position of the sensitivity multiplier, the transmittance reading is changed by a factor precisely equal to $\sqrt{10}$ and the absorptance reading is changed by 0.5 absorbance (optical density) unit. In this manner all readings can be made at nearly the same meter deflection, effectively compensating for any possible small nonlinearity error of the meter scale.

Thus the upper limits of the recommended ranges are 160 and 200 mg. urea N per 100 ml. for "S" tube and Coleman cuvette, respectively, when the Beckman Model B spectrophotometer is used.

*Per cent Relative Analysis Error per 1 Per Cent Absolute Photometric Error.**—The relative analysis error for a 1 per cent absolute photometric error is obtained by the method shown by Ayres.[3] This value has been found to be 3.1 for curve 1, and 2.6 for the modification described, curve 2, Fig. 4.

SUMMARY

An accurate microcolorimetric determination of urea using alpha-iso-Nitrosopropiophenone has been described. The method has been found to be adequate for clinical investigations since it is rapid as well as accurate. The range recommended for urea determination is 8 to 160 mg. of urea N per 100 ml. The per cent relative analysis error per 1 per cent absolute photometric error has been reduced to 2.6 from the previously reported 3.1.

A practical application of an overflow micropipette is described. It is useful in nearly all microchemical procedures in quantitative clinical chemistry.

The author is indebted to Dr. Oliver H. Lowry for the suggestion that urease might be used to establish the precision of the method.

And he also takes pleasure in acknowledging the technical assistance given by Miss Harriette M. McCarthy.

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*It is obtained by dividing 230 by the slope of the curve, which is the absorptancy change in per cent, corresponding to one logarithmic cycle on the concentration axis—i.e., a tenfold change in concentration. For a detailed discussion of this subject, an excellent article by Ayres[3] is highly recommended.

THE CHEMICAL DETERMINATION OF TOCOPHEROLS IN FECES, AND THE FECAL EXCRETION OF TOCOPHEROL IN MAN

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THE factor of intestinal absorption has received little attention in studies on tocopherol. In a few instances it has been investigated indirectly by means of tolerance tests based on plasma tocopherol curves,[1-5] or by means of bio-assays of total body tissue,[6] but both methods have proved unsatisfactory. Recently it has been shown that the results of tocopherol tolerance tests depend on several factors, and that they do not necessarily reflect the rate or magnitude of absorption.[7] Bio-assays or chemical analyses of total body tissue, on the other hand, provide a crude index of tocopherol absorption, but the results cannot be quantitated, since no distinction can be made between the losses of tocopherol due to utilization in the tissues and those due to incomplete absorption.

Balance studies, based on chemical analyses of the feces and measurements of the intake, would appear to offer the best means of estimating tocopherol absorption. The validity of this method is based on two assumptions, that there are no significant losses of tocopherol by excretion in the urine or by destruction in the intestinal tract, and that there is no significant enteric synthesis of tocopherol. Previous investigators[7, 8] have clearly demonstrated that little tocopherol is excreted in the urine. The evidence that none is destroyed in the intestinal tract, however, is based on in vitro incubation experiments with aspirated intestinal contents and feces,[7] and still needs further confirmation by more direct methods. The possibility that tocopherol is synthesized in the intestinal tract has not been ruled out, but appears unlikely. The only contradictory evidence[9] is based on the observation that the feces of rats on stock rations have significant vitamin E activity. This cannot be interpreted as evidence of tocopherol synthesis, since it is based on the assumption that dietary tocopherol is completely absorbed in the intestinal tract, an assumption which is not borne out by the results of other investigations.[7, 10] There is evidence to suggest that some of the tocopherol absorbed is re-excreted into the intestinal tract via the biliary tract.[7] How much is reabsorbed, and to what extent the biliary component affects the total fecal excretion of tocopherol, especially in diseased states, requires further investigation. Nevertheless, the authors have recently presented evidence indicating that balance studies based on fecal analyses do provide a satisfactory means of measuring tocopherol absorption.[7]

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There have been few other attempts to study the fecal excretion of tocopherol. In 1940 Cuthbertson, Ridgeway, and Drummond[11] reported that rat feces contained no tocopherol when the diet was adequate with respect to vitamin E, but that as much as 25 per cent of the intake was excreted when a large supplement of wheat germ oil was added. The spectroscopic method employed by these investigators was relatively insensitive, requiring a minimum concentration of 0.1 per cent for detection, so that the presence of small amounts of tocopherol in the feces of rats on stock rations could not be excluded. Hines and Mattill[8] confirmed the presence of tocopherol in the feces of rats on a high tocopherol intake, employing florisil adsorption and the Emmerie-Engel ferric chloride-reduction bipyridine reaction, but did not quantitate their results. Engel and Heins,[10] using a similar method, appear to have been the only investigators to apply the technique of fecal analysis to the study of tocopherol absorption. They found that rats excreted 87 per cent of the tocopherol contained in dry grass, and only 37 to 44 per cent of that in wheat germ oil, and concluded that tocopherol was better absorbed from the latter. Some years later Engel[12] reported that the method employed in these rat studies did not yield satisfactory results with human feces, and could not, therefore, be used for absorption experiments in man.

In 1946 Quaife and Harris[13] described a highly sensitive method for the quantitative separation of tocopherols from lipids, based on the technique of molecular distillation under low pressure. Total tocopherols were determined colorimetrically by the Emmerie-Engel reaction following preliminary hydrogenation to remove interfering carotenoids. As little as 60 micrograms of tocopherol could be recovered quantitatively from one gram of fat. Later the method was applied to the analysis of foods[14] and tissues,[15] and its validity as a measure of vitamin E activity established by comparing the results with those of bio-assay.

The present report concerns the application of Quaife's distillation technique to the determination of total tocopherols in feces. With minor modifications it has proved a highly satisfactory method for this purpose, and has provided a means for studying tocopherol absorption in man.[7]

In balance studies involving the use of test doses of tocopheryl acetate, it is essential that the presence of the unhydrolyzed ester in the feces be excluded. Since the esters of tocopherol do not react in the Emmerie-Engel reaction, they escape detection by the distillation method described and must be demonstrated by other means. A method for this purpose, based on saponification in a closed system under nitrogen to prevent oxidation, is described. While inferior to the distillation method for determining free tocopherols, it is sufficiently accurate, when carried out in conjunction with the latter, to detect the presence of esters in the feces.

METHODS

Reagents.—

1. *Absolute ethyl alcohol.*
2. *Skellysolve B:* This must be repurified to eliminate interfering reducing substances.

To a 3-liter glass-stoppered separatory funnel add 1 liter of crude Skellysolve B, 120 ml. of 4 per cent NaOH, and 60 ml. of 13 per cent $AgNO_3$. Shake for six minutes, and allow to separate. Discard the lower layer and dry the remaining supernatant in a large flask over a layer of anhydrous $CaCl_2$ for a period of at least two hours (stopper with an aluminum-foil-covered cork and shake occasionally). Distill the clear supernatant over a water-bath at 40 to 70° C., using an all-glass apparatus with foil-covered cork connections. Discard the first 100 ml. of distillate and collect the remainder in a new receiver. Store in a brown glass bottle containing iron wire to retard peroxide formation.

Test the purity of the distillate as follows: evaporate 200 ml. of the Skellysolve B to dryness over a water bath (40 to 70° C.), and then take up the residue in 10 ml. of absolute ethyl alcohol. Carry out the Emmerie-Engel reaction, as described below, on an 8 ml. aliquot. The amount of reducing material present should not exceed the equivalent of 2 μg of tocopherol.

3. *Alpha, alpha'-bipyridine:* 1.0 Gm. in 200 ml. of absolute ethyl alcohol. Store in a dark bottle.

4. *Ferric chloride hexahydrate ($FeCl_3.6H_2O$):* After grinding in a mortar, dissolve 0.5 Gm. in 250 ml. of absolute ethyl alcohol. Store in a dark bottle.

5. *Palladinized calcium carbonate catalyst, 5 per cent:* Obtained from Baker and Co., Inc., Newark, N. J.

Standards.—

1. *Stock standard:* 500 mg. of pure, natural d-alpha-tocopherol (Distillation Products, Inc., Rochester, N. Y.) made up to 500 ml. with absolute ethyl alcohol. Store in a dark bottle at –20° C.

2. *Working standard:* 5 ml. of stock standard made up to 200 ml. with absolute ethyl alcohol (1 ml. contains 25 μg of tocopherol). Store in a dark bottle at 4° C. when not in use.

Special Precautions.—All glassware used should be washed in water, rinsed with C.P. acetone, using an all-glass blow bottle, and air dried. Do not use ethyl ether, which often contains peroxides which destroy tocopherol, and other impurities which give a color with the Emmerie-Engel reagents.[16] In none of the procedures should the petroleum ether solvent be permitted to come in contact with rubber, since it may extract Emmerie-Engel reacting materials. Use glass stoppers and inert silicone grease, aluminum-foil-covered corks, and polyethylene tubing.

Collection and Storage of Feces.—For balance studies it is recommended that three-day collections be made, the periods being marked off with carmine-red capsules in the usual manner. Individual specimens may be stored in waxed cartons at ordinary refrigerator temperatures until the entire collection has been completed, without significant loss of tocopherol. In fact, it has been found that there is no significant loss over a period of forty-eight hours at temperatures as high as 37° C. Feces may be stored for longer periods at –20° C.; however, it is recommended that homogenization be carried out first.

Homogenization.—After weighing the total collection (A) transfer the feces quantitatively to a Waring blendor and homogenize for fifteen to twenty minutes. If the feces are relatively dry and good homogenization is not achieved within five minutes, add a measured volume of water (B) and continue homogenization until a thin, smooth suspension is obtained. Usually an addition of water equivalent to 10 to 20 per cent of the original weight of the feces will suffice to accomplish this.

Freezing and Dehydration.—Weigh out 10-gram aliquots (C) of the fecal homogenate into each of four tared 250 ml. centrifuge bottles and shell-freeze in a mixture of acetone and dry ice or in a Deepfreeze.

Attach the bottles to a vacuum line and dry to constant weight at room temperature. The dried weights (D) of the individual specimens should check within 50 milligrams. The dehydrating apparatus used in this laboratory consists of a heavy-duty, mechanical vacuum pump connected in series with a cold trap, a 2-liter drying bottle lying on its side and half-filled with $CaCl_2$, and a glass manifold with outlets for eight rubber hose connections. With this apparatus complete dehydration is achieved within eighteen hours.

The dried feces in all four bottles are scraped out into a small Erlenmeyer flask with a metal spatula, corked and stored at –20° C.

Lipid Extraction.—The extraction apparatus used in this laboratory is similar to that described by Quaife and Harris,[14] but has been simplified by omitting the three-legged extraction cup and substituting a double row of glass pegs, made by invaginating the wall of the extraction flask, to hold the Soxhlet thimble securely in place.

To each of two weighed Soxhlet thimbles, transfer approximately one-half of the previously prepared dried feces. Reweigh and record the weight of each sample (E). Seat each thimble in an extracting flask containing 50 ml. of absolute ethyl alcohol and reflux over a boiling water bath for eighteen hours. After allowing the flask to cool, rinse the condenser, and then the upper portion of the flask and thimble with 15 ml. of absolute ethyl alcohol.

Discard the thimble, cool the flask in cold running water, and add a pinch of anhydrous sodium sulfate, 30 ml. of distilled water, and 100 ml. of Skellysolve B (F). Where low concentrations of tocopherol are suspected, or where the weight of dried feces in the thimble (E) is low (e.g., less than 2 Gm., instead of the usual 4 to 5 Gm.) reduce the volume of Skellysolve B to 50 milliliters. Insert a greased stopper and shake for fifteen minutes (preferably in a mechanical shaker).

After allowing the layers to separate, transfer the clear supernatant petroleum ether extract to a small tightly corked flask, and store at –20° C. until ready for distillation or saponification.

Molecular Distillation.—The details of the construction and operation of the required apparatus may be found in Quaife's papers[13, 14] and need not be reiterated. The molecular pump and Pirani gauge are obtainable from the Vacuum Equipment Division, Distillation Products, Inc., Rochester, N. Y., but the remaining parts must be made up by a local glass-blower and machinist.

Operation and maintenance are greatly facilitated if the parts are firmly mounted on a heavy 2-inch baseboard, and securely braced within an adjustable frame (Flexaframe rods and joints, Fisher and Company, New York, N. Y.). Of several methods tried for joining the various units of the apparatus, tapered ground-glass joints have proved most satisfactory. The joints are lightly greased, any excess being wiped off with acetone, wrapped tightly and carefully with several layers of overlapping Scotch Pressure Sensitive Tape, No. 470 (Minnesota Mining and Manufacturing Co., St. Paul, Minn.), and then shellacked. This provides an excellent vacuum seal, is readily removed with a razor blade, and allows enough play in the joints to prevent breakage as a result of minor jars.

A thermostatically-controlled oil-bath, operated by a 1000-watt flexible heating cable, is readily constructed of heavy copper and is mounted on a scissors-type automobile jack with a short handle for ease in raising and lowering during distillation.

A point of considerable importance in the assembly of the still not mentioned in her original paper,[13] but called to our attention by Quaife,[17] is the distance between the bottoms of the stills and the condensers. In the diagram accompanying Quaife and Harris' paper[13] a distance of 27 mm. is indicated. However, the condenser in each still must be adjusted by trial and error until maximum (usually quantitative) recoveries of tocopherol are attained.

As originally reported by Quaife and Harris[13] molecular distillation of tocopherol requires a pressure of 1 micron or less. However, it has been found in this laboratory that it is not possible to attain a pressure below 4 microns with a small single-stage molecular pump within any reasonable length of time. On communicating with the authors[17] it was learned that pressures as high as 10 microns had been found satisfactory. Since then all distillations have been carried out at 6 or 7 microns.

A 10 ml. aliquot (G) of the previously prepared Skellysolve B extract is transferred to a clean aluminum distillation cup. The petroleum ether is then evaporated under a stream of nitrogen over a slightly warmed electric plate; the cup is rocked from side to side to obtain an even coating of lipid on the bottom, leaving as little as possible on the sides.

The cup is transferred to the still and distilled for 30 minutes at a temperature of 215 to 220° C. and a pressure of 6 to 7 microns. The distillate which adheres to the bottom of the condenser is then transferred directly to alcohol by carefully immersing and rotating the lower end of the condenser in 50 ml. of absolute ethyl alcohol (H) contained in a small beaker. The solution of the distillate in alcohol is facilitated by adding 100 ml. of warm water (70° C.) to the interior of the condenser immediately after removing the dry ice and acetone "cold-finger."

Tocopherol Determination.—The procedure is essentially that of the Quaife and Biehler modification[18] of the Quaife and Harris method for plasma tocopherol.[19] The details of the hydrogenation technique and a description of the apparatus can be found in Quaife and Biehler's paper.[18]

Transfer a 15 ml. aliquot (I) of the alcoholic solution of the distillate to a 50 ml. centrifuge tube. Add a pinch of palladinized calcium carbonate catalyst and hydrogenate for one minute.

Following hydrogenation centrifuge for fifteen minutes at 2,500 r.p.m. Pipette 5 ml. aliquots (J) of the supernatant into each of two cuvettes and add 3 ml. of absolute ethyl alcohol and 1 ml. of alpha, alpha'-bipyridine reagent (a Coleman Jr. Spectrophotometer and 19 mm. cuvettes are used in this laboratory).

Pipette 8 ml. of absolute ethyl alcohol and 1 ml. of alpha, alpha'-bipyridine reagent into a third cuvette to serve as a *blank*.

Pipette 1 ml. each of working standard (containing 25 μg of tocopherol) and bipyridine reagent, plus 7 ml. of absolute ethyl alcohol into a fourth cuvette to serve as a *control*.

To the *blank* add 1 ml. of ferric chloride reagent and mix by twirling. With the wavelength of the spectrophotometer set at 520 millimicrons and the cuvette in place, set the light transmission at 100 per cent exactly fifteen seconds after the last drop of ferric chloride has been added.

Now add ferric chloride to the *control* in a similar manner and determine the light transmission. The reading should coincide exactly with that previously obtained with 25 μg of tocopherol in preparing the calibration curve. This serves as a control on the reagents and technique.

Repeat the above procedure with the sample, and determine the content of tocopherol (K) from the previously prepared calibration curve.

If the galvanometer reading is too low to read accurately, repeat the Emmerie-Engel reaction with a smaller aliquot (J) of the hydrogenated alcoholic solution of distillate (I), diluting to a volume of 8 ml. with absolute ethyl alcohol.

A fresh 25 μg control should be run with each pair of samples.

The *calibration curve* is prepared by carrying out the Emmerie-Engel reaction on 1, 2, 3 and 4 ml. aliquots of working standard (25, 50, 75, and 100 μg, respectively) diluted to 8 ml. with absolute ethyl alcohol.

Calculation.—

$$\text{Milligram of total tocopherol present in fecal collection} = \frac{K}{1000} \times \frac{H}{J} \times \frac{F}{G} \times \frac{L}{E}$$

K = micrograms from calibration curve
H = volume of alcohol used to dissolve distillate
J = aliquot of H used in Emmerie-Engel reaction
F = volume of Skellysolve B used in extraction
G = aliquot of F used in distillation
E = weight of dried feces in Soxhlet thimble
L = dried weight of entire fecal collection

$$L = \frac{A + B}{C} \times D$$

A = original weight of fecal collection
B = weight of water added for homogenization
C = weight of fecal homogenate in *each* drying bottle
D = dried weight of C (*average* of four bottles)

When the volumes recommended above are used, the total tocopherol content of the feces

$$\text{in mg.} = \frac{K}{1000} \times \frac{50}{5} \times \frac{100}{10} \times \frac{(A + B)}{10E} \times D$$

Saponification Technique to Determine Tocopherol Esters.—Prepare a *0.1N solution of KOH in alcohol* by dissolving 2.805 Gm. of KOH (analytical grade) in approximately 400 ml. of warmed absolute ethyl alcohol. Cool to room temperature and dilute to 500 ml. with alcohol. Allow carbonates to settle, and decant clear supernatant into the alkali reservoir *A* (Fig. 1), a 1-liter bottle fitted with a greased, ground-glass plug *B*, into which are fused tubes *C* and *D*.

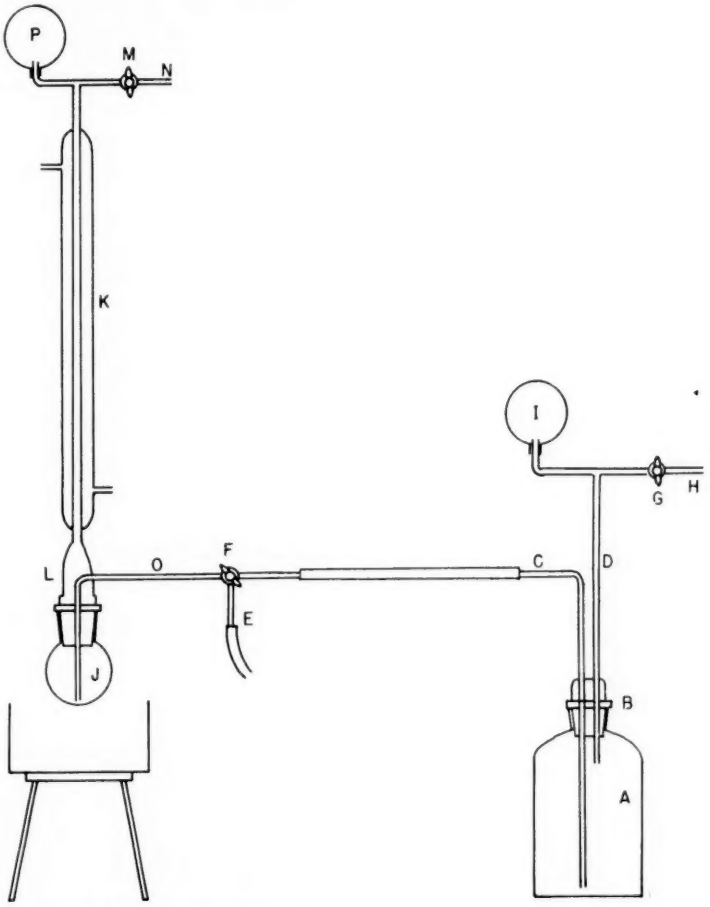

Fig. 1.—Apparatus for saponification of tocopheryl acetate.

Nitrogen is bubbled through the alkali by directing the gas in line *E* into tube *C* through the three-way T-stopcock *F*. Stopcock *G* is left open so that the nitrogen escapes through tube *H*. At the end of two hours stopcock *G* is closed and the balloon *I* is allowed to distend to a volume of approximately 500 ml. By closing stopcock *F* the alkali is maintained under a positive pressure of nitrogen, and may be used for a period of a week (provided there has been no leakage).

A 25 ml. aliquot of the Skellysolve B extract of the feces, obtained in the lipid extraction described above, is transferred to the 100 ml. round-bottomed saponification flask *J*, and is evaporated to dryness under a stream of nitrogen by gently heating in warm water. The lipid is redissolved in 5 ml. of absolute ethyl alcohol with the aid of gentle heating;

the flask is then attached to the reflux condenser *K* by means of the greased, ground-glass joint *L* and held firmly in place by means of rubber bands or springs attached to the glass ears on either side of the joint.

With stopcock *F* closed and stopcock *M* open, the saponification flask is evacuated of air through tube *N* by means of a mechanical vacuum pump and cold-trap for a period of five minutes.

The system is now washed out with nitrogen from line *E* by closing stopcock *M* and opening *F* so that the gas is directed through tube *O* which is fused into the neck of the reflex condensor and projects into flask *J*. Enough gas is run into the system to distend rubber balloon *P* to a volume of approximately 500 ml. This procedure of evacuation followed by washing out with nitrogen is repeated twice, and finally the system is evacuated and sealed by closing stopcocks *M* and *F*.

Approximately 2 ml. of the alkali in *A* is then transferred to the saponification flask by opening stopcock *F* momentarily so that the alkali is sucked into the flask through tube *O*.

The saponification flask is then immersed in a boiling water bath and its contents allowed to reflux for ten minutes. A slow stream of nitrogen is bubbled through the flask during saponification and is allowed to collect in balloon *P*.

Cool the flask in a pan of cold water, release the nitrogen pressure by opening stopcock *M* and then detach from the condenser. Add 5 ml. of water, a pinch of anhydrous sodium sulfate and 15 ml. of Skellysolve B. Shake for fifteen minutes after having sealed the flask with a greased, ground-glass stopper. Allow to stand until the layers separate and then transfer 10 ml. of the petroleum ether layer to a 50 ml. centrifuge tube.

Evaporate to dryness under a stream of nitrogen by gentle heating, and then redissolve the remaining lipid in 10 ml. of absolute ethyl alcohol. Hydrogenate and carry out the Emmerie-Engel reaction in an 8 ml. aliquot of alcohol as described in the distillation method.

Calculation.—Total tocopherol including esters in fecal collection, in milligrams =

$$\frac{K}{1000} \times \frac{F}{25} \times \frac{15}{10} \times \frac{10}{8} \times \frac{L}{E}$$

K = micrograms from calibration curve
F = volume of Skellysolve B used in original extraction
L = dried weight of feces of entire fecal collection
E = weight of dried feces in Soxhlet thimble

Tocopherol esters (expressed as free tocopherol) present in fecal collection equals tocopherols by saponification minus tocopherols by distillation.

RESULTS

Recoveries of Tocopherol Following Molecular Distillation.—Almost quantitative recoveries (99.8 ± 1.5 per cent) were obtained following distillation of alcoholic solutions of pure, d-alpha-tocopherol in amounts ranging from 10 to 1000 μg (Table I). The mean difference between duplicate determinations was less than 1 per cent, and did not exceed 4 per cent in any instance.

When tocopherol was added to 100 Gm. aliquots of feces, in amounts ranging from 10 to 1000 mg., and then subjected to homogenization, dehydration, lipid, extraction, and distillation, recoveries of 92.0 ± 8.4 per cent were achieved (Table II). Somewhat better recoveries (96.3 ± 2.5 per cent) were attained in the range of concentrations usually encountered in balance experiments (25 to 1,000 mg. per 100 Gm. of feces). The excessive losses noted with additions of only 10 mg. of tocopherol were probably related to poor admixture with the feces.

No free tocopherol was demonstrable by the Emmerie-Engel reaction following molecular distillation of dl-alpha-tocopheryl acetate in alcohol solu-

TABLE I. RECOVERY OF D-ALPHA-TOCOPHEROL IN ALCOHOL

TOCOPHEROL IN SAMPLE	RECOVERED BY DISTILLATION*		RECOVERED BY SAPONIFICATION	
MG.	MG.†	PER CENT	MG.†	PER CENT
0.010	0.0101	101.0		
0.010	0.0101	101.0		
0.025	0.0258	103.2	0.0248	99.2
0.025	0.0248	99.2	0.0238	95.2
0.025			0.0275	110.0
0.025			0.0339	135.6
0.050			0.0385	77.0
0.050			0.0385	77.0
0.050			0.0439	87.8
0.050			0.0491	98.2
0.075			0.0619	82.5
0.075			0.0716	95.5
0.075			0.0666	88.0
0.075			0.0600	88.0
0.100	0.0994	99.4	0.0876	87.6
0.100	0.1006	100.6	0.0936	93.6
0.100			0.0705	70.5
0.100			0.0820	82.0
0.500	0.4937	98.7	0.4530	90.6
0.500	0.4912	98.2	0.4220	84.4
0.500			0.4640	92.8
1.000	0.9833	98.3	0.9100	91.0
1.000	0.9817	98.2	0.8920	89.2
1.000			0.9240	92.4
5.000			4.5700	91.4
5.000			4.3400	86.8
5.000			4.6800	93.6
10.000			9.1200	91.2
10.000			9.0200	90.2
10.000			8.9400	89.4
100.000			96.3400	96.3
100.000			96.0220	96.0
100.000			96.4320	96.4
Mean ± s.d.		99.8 ± 1.5		91.6 ± 10.1

*Solution of d-alpha-tocopherol in absolute ethyl alcohol added to distillation cup, evaporated by heat in a stream of nitrogen and distilled; duplicate samples distilled simultaneously in separate stills.

†Values recorded are the average results of duplicate Emmerie-Engel reactions carried out on each sample; the mean difference between such duplicates was 2.8 per cent, and did not exceed 7.1 per cent in any instance.

tions, in amounts up to 100 μg (Table III), or in fecal hemogenates, in amounts up to 1,000 milligrams.

Recoveries of Tocopherol Following Saponification.—The losses following saponification of d-alpha-tocopherol were significantly greater than those following distillation, both in alcoholic solutions (Table I) and in fecal homogenates (Table II), especially at low concentrations. Thus recoveries of only 91.6 ± 10.1 and 87 ± 5.8 per cent, respectively, were achieved. Moreover, the results were not nearly as reproducible, the difference between replicate determinations averaging 10.5 per cent and ranging from 0.1 to 29.8 per cent (Table I).

TABLE II. RECOVERY OF D-ALPHA-TOCOPHEROL ADDED TO FECES*

TOCOPHEROL CONTENT OF FECES BY DISTILLATION (A)	TOCOPHEROL CONTENT OF FECES BY SAPONIFICATION (B)	TOCOPHEROL ADDED TO FECES (C)	TOCOPHEROL RECOVERED			
			BY DISTILLATION		BY SAPONIFICATION	
			AMOUNT (D)	PER CENT $\frac{(D)-(A)}{(C)} \times 100$	AMOUNT (E)	PER CENT $\frac{(E)-(B)}{(C)} \times 100$
MG.	MG.	MG.	MG.		MG.	
14.74	11.22	10.00	23.65	89.1	—	—
12.42	9.75	10.00	19.73	73.1	17.63	78.8
12.42	9.75	25.00	35.52	92.4	30.36	82.4
12.42	9.75	50.00	59.62	94.4	51.62	83.7
12.42	9.75	100.00	110.24	97.8	100.97	91.2
12.42	9.75	500.00	504.42	98.4	471.66	92.4
12.42	9.75	1000.00	998.62	98.6	951.82	94.2
				92.0 ± 8.4†		87.1 ± 5.8†

*Solution of d-alpha-tocopherol in absolute ethyl alcohol added to 100 Gm. aliquots of homogenized feces before dehydration and lipid extraction. Values recorded are the averages of duplicate determinations.

†Omitting the experiments with 10 mg. additions of tocopherol, the recoveries were 96.3 ± 2.5 per cent following distillation and 88.8 ± 4.8 per cent following saponification.

However, in analyses of tocopheryl acetate the distillation method failed completely, while saponification yielded recoveries of 89.3 ± 8.6 per cent in alcoholic solutions (Table III) and 76.2 ± 3.6 per cent in fecal homogenates (Table IV).

Tocopherol Content of Feces in Man.—The daily excretion of tocopherol, as measured by molecular distillation, was 21.62 ± 2.90 mg., in normal healthy adults, or an average of 64.4 per cent of the calculated dietary intake, and 16.43 ± 2.81 mg., or 53.5 per cent of the intake in hospitalized convalescents. Subjects with Laennec's cirrhosis, on the other hand, excreted significantly less, 7.41 ± 2.99 mg., or 35.2 per cent of the intake (Table V), and evidence was adduced to show that this was the consequence of more complete absorption of dietary tocopherol.[7]

Duplicate determinations were in close agreement, the difference in fifty successive analyses averaging 1.4 per cent, and not exceeding 10.7 per cent in any instance.

When feces were analyzed for free tocopherol by both methods, saponification invariably yielded results 20 to 30 per cent lower than distillation (Table VI). It will be noted that almost identical losses were encountered when fecal homogenates containing added tocopheryl acetate were saponified (Table IV). In an attempt to reduce these losses a number of other techniques for hydrolyzing tocopheryl esters were tried, but none yielded as good results as saponification in a closed system under nitrogen.

Obviously saponification is inferior to molecular distillation for the accurate determination of free tocopherol in feces, but when carried out in conjunction with distillation may be used to estimate the content of tocopheryl esters, provided due allowance is made for the expected 20 to 30 per cent loss, and it is recognized that small amounts may escape detection.

In a number of balance experiments, in which large doses of tocopheryl acetate were administered, no esterified tocopherol could be demonstrated in the feces. The results of two representative experiments are outlined in Table

TABLE III. RECOVERY OF DL-ALPHA-TOCOPHERYL ACETATE IN ALCOHOL

TOCOPHEROL IN SAMPLE		FREE TOCOPHEROL RECOVERED			
		BY DISTILLATION†		BY SAPONIFICATION	
ACETATE MG.	FREE* MG.	MG.	PER CENT	MG.	PER CENT
0.025	0.0229	0.000	0.0	0.0173	75.5
0.025	0.0229	0.000	0.0	0.0219	97.3
0.050	0.0458	0.000	0.0	0.0386	84.2
0.050	0.0458	0.000	0.0	0.0368	80.4
0.075	0.0686	0.000	0.0	0.0660	96.3
0.075	0.0686	0.000	0.0	0.0534	77.9
0.100	0.0914	0.000	0.0	0.0610	66.7
0.100	0.0914	0.000	0.0	0.0700	77.1
0.500	0.4570			0.3450	75.5
0.500	0.4570			0.3300	72.2
1.000	0.9140			0.8000	87.5
1.000	0.9140			0.8400	91.9
1.000	0.9140			0.8140	89.1
1.000	0.9140			0.8260	90.4
5.000	4.5700			4.2000	91.9
5.000	4.5700			4.0000	87.5
5.000	4.5700			4.4000	96.3
5.000	4.5700			4.1500	90.8
10.000	9.1400			9.1000	99.6
10.000	9.1400			8.5000	93.0
10.000	9.1400			7.6000	83.2
10.000	9.1400			8.0000	87.5
50.000	45.7000			43.3000	94.7
50.000	45.7000			43.4000	95.0
50.000	45.7000			44.1000	96.5
50.000	45.7000			42.0000	91.9
100.000	91.4000			88.0000	96.3
100.000	91.4000			84.0000	91.9
100.000	91.4000			82.0000	89.7
100.000	91.4000			90.0000	98.5
500.000	457.0000			452.0000	98.9
500.000	457.0000			440.0000	96.3
500.000	457.0000			460.0000	100.7
500.000	457.0000			432.0000	94.5
Mean ± s.d.			0.0		89.3 ± 8.6

*1 mg. dl-alpha-tocopheryl acetate contains 0.914 mg. free tocopherol.

†dl-alpha-tocopheryl acetate in absolute ethyl alcohol added to distillation cup, evaporated by heat under a stream of nitrogen and distilled.

VII. It will be noted that as the dose of tocopheryl acetate was increased there was a proportional increment in the tocopherol content of the feces as determined by the distillation method. However, the percentage of distilled tocopherol recovered following saponification remained constant, from which it may be inferred that esters were not present. Evidence has been presented elsewhere[7] to show that normally tocopheryl esters are rapidly split by esterases in the intestinal tract. It is conceivable, however, that under certain pathological conditions tocopheryl esters might pass through the intestinal tract unchanged.

TABLE IV. RECOVERY OF ADDED DL-ALPHA-TOCOPHERYL ACETATE* IN FECES BY SAPONIFICATION

TOCOPHEROL CONTENT OF SAMPLE BY DISTILLATION†	TOCOPHEROL CONTENT OF SAMPLE BY SAPONIFICATION† (A)	TOCOPHERYL ACETATE ADDED		TOCOPHEROL FOUND BY SAPONIFICATION (C)	RECOVERY $\frac{(C)-(A)}{(B)} \times 100$
		ACETATE	FREE (B)		
MG.	MG.	MG.	MG.	MG.	PER CENT
14.74	11.22	10.00	9.14	18.17	76.0
8.64	6.84	10.94	10.00	13.94	71.0
8.64	6.84	10.94	10.00	15.16	83.2
8.64	6.84	27.35	25.00	26.23	77.6
8.64	6.84	27.35	25.00	24.39	70.2
8.64	6.84	54.70	50.00	42.68	71.7
8.64	6.84	54.70	50.00	45.00	76.3
8.64	6.84	109.40	100.00	84.26	77.4
8.64	6.84	109.40	100.00	82.10	75.3
8.64	6.84	547.00	500.00	395.40	77.7
8.64	6.84	547.00	500.00	391.08	76.8
8.64	6.84	1094.00	1000.00	796.60	79.0
8.64	6.84	1094.00	1000.00	791.88	78.5
					76.2 ± 3.6

*dl-alpha-tocopheryl acetate in absolute ethyl alcohol added to 100 Gm. aliquots of homogenized feces before dehydration and lipid extraction.

†Average of quadruplicate determinations (maximum deviation from the mean 0.04 mg).

TABLE V. FECAL EXCRETION OF TOCOPHEROL IN MAN AS DETERMINED BY THE MOLECULAR DISTILLATION METHOD

	HEALTHY ADULTS	HOSPITALIZED CONVALESCENTS	CIRRHOTICS
Daily* excretion, mg.	16.13	12.22	4.09
	19.98	15.55	4.17
	21.50	16.01	6.12
	23.27	17.51	7.54
	24.03	20.86	10.81
	24.79		11.74
average ± s.d.	21.62 ± 2.90	16.43 ± 2.81	7.41 ± 2.99
Average daily dietary intake of tocopherol, mg.†	33.53	30.76	21.03
Average percentage of ingested tocopherol excreted in feces	64.4	53.5	35.2

*Based on analyses of three-day stool collections in 6 healthy adults, five convalescents with no evidence of liver disease, and six subjects with Laennec's cirrhosis.

†The healthy adults were members of the staff who ate in the hospital dining room, the convalescents were on the routine ward diet and the cirrhotics were on a special measured diet. The average tocopherol content of the diets on five randomly selected days was estimated from the tables of Harris, Quaife, and Swanson.[20]

TABLE VI. COMPARISON OF FECAL CONTENT OF TOCOPHEROL AS DETERMINED BY BOTH THE DISTILLATION AND SAPONIFICATION TECHNIQUES IN SUBJECTS ON A NORMAL DIET WITHOUT TOCOPHEROL SUPPLEMENTS

TOCOPHEROL BY DISTILLATION	TOCOPHEROL BY SAPONIFICATION	PER CENT OF TOCOPHEROL FOUND BY DISTILLATION RECOVERED FOLLOWING SAPONIFICATION
MG.	MG.	
8.64	6.84	79.2
12.42	9.75	78.5
14.74	11.22	76.1
16.21	12.01	74.1
23.27	16.92	72.7
	Mean ± s.d.	76.1 ± 2.5

TABLE VII. RECOVERY OF TOCOPHEROL FROM FECES IN SUBJECTS INGESTING LARGE AMOUNTS OF DL-ALPHA-TOCOPHERYL ACETATE

SUBJECT	PERIOD (3 DAYS)	DL-ALPHA-TOCOPHERYL ACETATE INGESTED	TOCOPHEROL RECOVERED BY DISTILLATION	TOCOPHEROL RECOVERED BY SAPONIFICATION	
		MG.	MG.	MG.	PER CENT OF AMOUNT RECOVERED BY DISTILLATION
Normal M. W.	I	0	69.81	50.76	72.7
	II	1700	1138.80	916.62	80.5
	III	1800	1115.92	807.53	72.4
	IV	500	429.51	321.44	74.8
	V	0	72.99	53.40	73.2
	VI	0	65.79	47.03	71.5
Normal S. L.	I	0	48.62	36.02	74.1
	II	1700	954.76	726.64	76.1
	III	1800	1150.12	877.31	76.3
	IV	500	354.45	259.90	73.3
	V	0	58.19	41.55	71.4
	VI	0	55.30	40.43	73.1

The distribution of the alpha, beta, gamma, and delta forms in the total tocopherols of the feces has not been investigated, although such studies might be important in interpreting the physiological significance of fecal losses of tocopherol.

SUMMARY

A method for determining total tocopherols in feces, based on molecular distillation and the Emmerie-Engel reaction is described, and values for the daily excretion in man are reported.

Tocopheryl esters escape detection in the Emmerie-Engel reaction, so that a technique for preliminary release of free tocopherol, based on saponification in a closed system under nitrogen has been devised. When used in conjunction with the distillation method it provides a satisfactory means for determining tocopheryl esters in feces. No tocopheryl esters have been demonstrated in the feces of individuals receiving large doses of tocopheryl acetate.

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SCINTILLATION EQUIPMENT FOR USE IN THE DETECTION OF RADIOACTIVITY IN THE LOCALIZATION OF INTRACRANIAL LESIONS

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THE recent interest in the use of radioactive diiodofluorescein[1, 2] and, more recently, iodinated human albumin[3] in the localization of intracranial lesions emphasizes the need for sensitive, directional equipment for the quantitative determination of radioactivity within the cranium. In our experience the scintillation equipment herein described has enabled us to use much less radioactivity (50 to 100 microcuries of diiodofluorescein instead of 1 millicurie) and provided us with better resolution than is possible with the Geiger counter apparatus previously described.

APPARATUS

The detector used in his work is a scintillation counter consisting of a thallium activated sodium iodide crystal with a 5819 photomultiplier tube and preamplifier surrounded by a lead collimator (Fig. 1). Collimation is achieved by a lead tube 5 cm. in diameter, 7.5 cm. long and a 2.5 cm. bore faced with ⅜ inch of sponge rubber. A Mu-metal shield is necessary to protect the 5819 tube from the earth's magnetic field since it was found that the counting rate of a standard varied by a factor of 2 when the detector was rotated without this shield. The entire detector is housed in an aluminum tube 12 inch long and 3 inch in outside diameter. This provides the sodium iodide crystal and 5819 tube with at least one-half inch of lead shielding in all directions.

The crystal ¾ inch in diameter and 1 inch long, is sealed in oil in a lucite container. This lucite cartridge is spaced approximately ⅛ inch from the face of the 5819 tube using "O" rings to give an oil and light tight seal. The space between the lucite and the tube is filled with oil using an intravenous needle inserted through the oil hole and then sealed. This reduces diffraction losses and increases the counting rates two to three times. The overall efficiency for this apparatus is 40 to 50 per cent for the I^{131} gamma radiation compared to approximately 0.5 per cent for conventional Geiger-Mueller tubes. It is this increased efficiency which permits the reduction in the amount of radioactivity administered. The resolution obtained with this apparatus is noted in Table I.

A diagram of the counter is recorded in Fig. 2. The output of the counter is fed into a suitable discriminator and to a counting rate meter adjusted to provide a prompt equilibrium rise. This is coupled to a 5 milliampere Esterline Angus recorded into which has been inserted a switch to provide a prompt zero followed by an abrupt rise when a reading is to be

TABLE I. COUNTS PER MINUTE FROM A 3.6 MICROCURIE SPHERE 2.5 CM. IN DIAMETER

RADIUS (CM.)	−60 DEGREES	−50 DEGREES	−40 DEGREES	−30 DEGREES	−20 DEGREES	−10 DEGREES	0 DEGREES	+10 DEGREES	+20 DEGREES	+30 DEGREES	+40 DEGREES	+50 DEGREES	09+ 90 DEGREES
5	110	120	399	1769	5219	7699	8569	7769	5257	1703	379	122	99
10		64	69	147	1286	3785	4544	3693	1234	139	74	59	
15				78	345	2045	2789	1991	351	69			
20					157	1114	1909	151					

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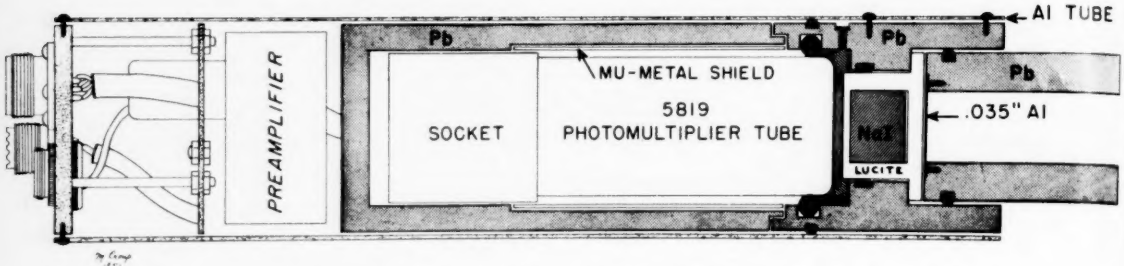

Fig. 1.—Collimated scintillation detector showing position of crystal, 5819 tube, preamplifier, and lead collimator.

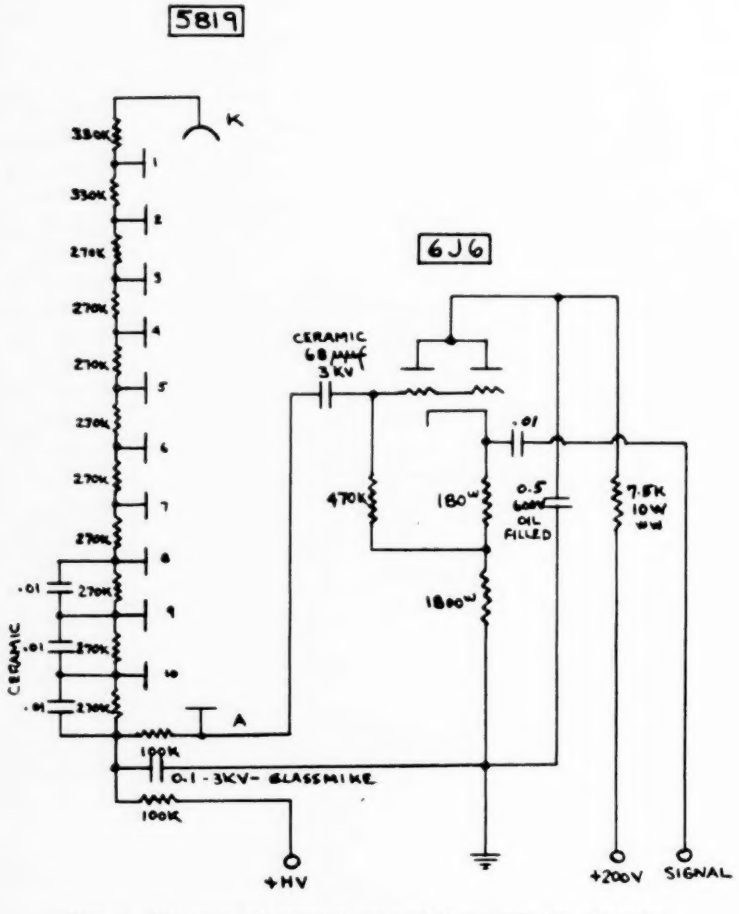

Fig. 2.—Diagram of counter used with scintillation detector.

taken. The entire counter is supported by a counter-balanced microphone boom. The collimating tube is held by a Quickset Sr. photographer's panning head and the stand for the above equipment is provided with Acme No. 658 ball type casters permitting flexible adjustment and rigid positioning of the tube in all positions about the skull.

SUMMARY

A directional scintillation counter suitable for use in the localization of intracranial lesions and permitting a smaller dose of radioactive material is described.

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STUDIES IN NONPROTEIN NITROGEN

II. Limitations of the Hypobromite Method in Measuring Nonprotein Nitrogen in Blood

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IN MOST clinical laboratories, the routine determination of blood nonprotein nitrogen is carried out in accordance with Folin-Wu's method of heat-digestion of an acidified blood filtrate, followed by nesslerization and photometric measurement.[1] A titrimetric micromethod, in which deamination with hypobromite solution replaces the digestion step, was recently described by Rappaport and Eichhorn as equal in accuracy to the Kjeldahl method and recommended by them for its rapidity.[2] However, because of contradictory statements in the literature concerning the hypobromite reaction,[3, 4] it was felt that before acceptance this method should be evaluated thoroughly for accuracy and reliability. It was therefore decided to compare the results obtained by both the hypobromite method and a modified Folin-Wu method with the nitrogen values determined by means of micro-Kjeldahl analysis.

MATERIALS AND METHODS

Specimens of oxalated blood and prepared urea solutions, both series representing a wide range of nonprotein nitrogen from normal to very high concentrations, were used in the study. The R method*[2] was followed in all essential details: three separate protein-free filtrates were made from each of ten test bloods by adding 1 ml. of blood to 25 ml. of "modified Abrahamson's reagent"; three 2-ml. aliquots from each of the 3 filtrates were treated with 5 ml. of the hypobromite solution. The resulting deamination was measured by titrating iodometrically the excess of bromine with a N/200 sodium thiosulfate solution which was stabilized by means of 0.01 Gm. of sodium carbonate per 100 ml. The titer of this solution was found to remain unchanged over a period of at least 4 weeks.†

In carrying out the F method‡[6] three tungstic acid filtrates were made from each of the same ten blood specimens; three aliquots from each of the three (F) filtrates were digested and nesslerized and their nitrogen content measured by comparison with that of ammonium sulfate standards in an Evelyn photoelectric colorimeter adapted with filter No. 515. The volume of filtrate subjected to digestion was 5 ml. with nitrogen contents in the normal range and was proportionately reduced with higher concentrations so that all photometric readings taken were within the range that conformed to Beer's law (40 to 80 per cent transmittance).

On each of these (R) and (F) filtrates, a micro-Kjeldahl analysis was performed, also in triplicate. In modifying the method as published,[7-9] 1 ml. of a 1.8 per cent selenium solution (prepared by heating powdered selenium in concentrated sulfuric acid until the

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*Abbreviations are explained in legend of Table I.

†Without sodium carbonate, a change in titer of the thiosulfate solution may readily occur[5] and, if undetected, cause a considerable error in nitrogen results.

‡Accidental losses slightly reduced these numbers in some cases.

TABLE I.—COMPARISON OF NONPROTEIN NITROGEN VALUES IN 10 BLOOD SAMPLES AS DETERMINED BY 3 METHODS

CLINICAL DIAGNOSES									
POOLED BLOOD	NORMAL	NORMAL	POOLED BLOOD	OBSTRUCTIVE UROPATHY‡	G. I. BLEEDING	UREMIA (?)	NEPHROSCLEROSIS	PYELONEPHRITIS	NEPHROSCLEROSIS
Mg. Nonprotein Nitrogen per 100 ml.—K (F) Method									
29.7	32.5	32.9	46.7	66.9	68.2	88.7	139.5	143.4	261.2
K (R) : K (F) Ratios									
0.95*	0.99	0.99	0.98	1.01	0.96*	0.98	1.00	1.03†	0.99
F : K (F) Ratios									
0.94†	1.07	1.13†	1.07*	1.05†	1.02	1.03	0.99	1.05†	1.03†
R : K (R) Ratios									
0.87†	0.94*	0.94†	0.97*	0.85†	0.92†	0.87†	0.81†	0.85†	0.83†
R : F Ratios									
0.88†	0.87†	0.83†	0.89†	0.81†	0.87†	0.82†	0.81†	0.83†	0.80†

K = Micro-Kjeldahl.
R = Rappaport-Eichhorn.
F = Folin-Wu (modified).
(R) = Blood filtrate made according to R method.
(F) = Blood filtrate made according to F method.
*Denotes ratios of values which were significantly different at the 0.05 probability level.
†Denotes ratios of values which were significantly different at the 0.01 probability level.
‡Same blood sample as first one in Table II.

final solution became deep green in color) and 4 ml. of a 12 per cent aqueous potassium sulfate solution were added to 5 ml. of the (R) filtrates or to 2 ml. of the (F) filtrates. The tubes were heated in a digestion rack until the water evaporated; digestion was maintained for thirty minutes after clearing. The digests were made alkaline with 5 ml. of 50 per cent aqueous sodium hydroxide and steam distilled through a Parnas-Wagner apparatus until 30 ml. of distillate were collected in a flask containing 10 ml. of a 4 per cent aqueous boric acid solution. Titration with 0.01N hydrochloric acid followed, using 5 drops of indicator until the color matched that of a blank which contained the same amounts of boric acid and indicator, and was diluted with distilled water to a volume equal to that of the distillate. The indicator consisted of three parts of 0.1 per cent bromcresol green in 95 per cent alcohol and two parts of 0.1 per cent methyl red in 95 per cent alcohol,[5, 10] and provided a very distinct end point by a sharp change from blue to red at a pH of 5.1.

Thus, nine* replicates each were determined by the R and F methods and eighteen* by Kjeldahl analysis on each of ten blood samples (Table I). Besides, in one of these, and in five additional blood samples, results of the R method as applied to the original (R) filtrates were compared with those obtained from diluted (R) filtrates after multiplying with the dilution factor (Table II). These analyses were performed in triplicate on single filtrates only. Similarly, triplicate readings were determined by each of the three methods on each of five urea solutions (Table III). These were treated in essentially the same manner as blood, except that distilled water was substituted for tungstic acid in making the tenfold dilutions for the F procedure; these dilutions were also utilized for K analysis.

Significance tests for comparison of results yielded by the three methods were carried out in accordance with the analysis of variance technique as originally described by Fisher.[11] Incidental to these analyses, estimates of the experimental error variances were obtained for each method. The square roots of these estimated values were computed to yield the standard experimental error. In the blood analyses, this standard error for the K method was 2.15 on (R) filtrates and 2.69 on (F) filtrates; for the F method, it was found to be 1.80 and for the R method, 1.27. In the experiments with urea solutions, the standard error for the K method was 1.85, for the R method, 2.02 and for the F method, 2.35.

TABLE II. COMPARISON OF R VALUES AS DETERMINED IN DILUTED AND UNDILUTED (R) FILTRATES

FILTRATE DILUTIONS: PARTS OF H_2O ADDED PER PART OF FILTRATE	CLINICAL DIAGNOSES					
	OBSTRUCTIVE UROPATHY	CHRONIC GLOMERULONEPHRITIS	OBSTRUCTIVE UROPATHY	OBSTRUCTIVE UROPATHY	NEPHROSCLEROSIS	PYELONEPHRITIS
	Mg. Nonprotein Nitrogen per 100 ml.—K (R) Method)					
	67.5	88.1	93.4	95.4	120.8	137.6
	R : K Ratios*					
0	0.85	0.89	0.96	0.90	0.86	0.89
1	0.99	0.90		0.92		0.94
2					0.91	
3			0.98			0.99
5					0.93	

*Arithmetic means of triplicates are listed.

TABLE III. COMPARISON OF NITROGEN VALUES IN UREA SOLUTIONS‡ AS DETERMINED BY THREE METHODS

Mg. N per 100 ml.—K Method				
21.5	42.2	82.6	164.7	327.6
F : K Ratios				
0.97	0.98	0.97	0.99	0.99
R : K Ratios				
0.92	0.93	0.94*	0.91†	0.78†

*†See Legend of Table I.

‡Prepared from a solution containing 0.7021 Gm. of pure urea (328 mg. of urea nitrogen) dissolved in 100 ml. of distilled water and diluted 2, 4, 8 and 16 times.

RESULTS

Comparison of (R) and (F) Filtrates by K Method.—The blood nonprotein nitrogen as measured by K analysis in (R) filtrates differed significantly from that yielded by the corresponding (F) filtrates on three of the ten blood specimens studied (Table I). In only one of these was the difference statistically significant at the 0.01 probability level, the K (R) : K (F) ratio being 1.03, while in the other two instances the difference was significant only on the 0.05 level, with ratios slightly less than one. Thus, since the direction of the deviation of the deviations was not consistent, it is apparent that there was no systematic bias between the two techniques—(R) and (F)—of preparing a protein-free filtrate. Consequently, any subsequent differences resulting from the application of the R and F methods must be inherent in the methods themselves.

Comparison of F and K Methods.—The F method gave, with two exceptions, somewhat higher results than the K analysis on the same blood filtrates, as illustrated by their ratios (Table I). These differences were, with one exception, quite small, although statistically significant in six of the ten blood samples examined. This tendency toward higher F results was quite consistent without predilection for any segment of the range of nitrogen concentrations studied. Hence, the geometrical mean of all ratios, calculated to be 1.04, may serve as a factor to convert F values to the approximate corresponding K values and vice versa. In the analysis of urea solutions, the relation between K and F values was reversed, but the differences were not significant (Table III).

Comparison of R and K Methods.—The R values were consistently lower than the K values found on the same blood filtrates, the differences being statistically significant in all ten examples so studied (Tables I and II). The ratios of R:K values showed marked variations, but were lowest at high nitrogen concentrations. This latter phenomenon was underscored in the urea experiments by the progressive decline of R:K differences with increasing nitrogen levels (Table III). Such an inequality in behavior between high and low nitrogen concentrations, in addition to the marked degree of variations, would appear to preclude the use of an over-all conversion factor.

Comparison of R and F Methods.—The relationship of R and F values was similar to that of R and K values, some of the differences in the case of blood filtrates being even greater since, as shown above, F values often exceeded the corresponding K (F) values.

Attempted Modifications of R Method.—Since the R method appeared to give better nitrogen recoveries at low nitrogen concentrations, it was applied to varying dilutions of the original (R) filtrates of six azotemic blood samples. Table II indicates that this procedure was effective in approximating results to the corresponding K values in each instance.

In other experiments, the time allowed for the hypobromite reaction, until the addition of hydrochloric acid and potassium iodide, was lengthened from the usual three minutes to five, eight, and ten minutes, respectively, in the hope of increasing the nitrogen recovery as suggested by Peters and Van Slyke.[3] However, no consistent change in results could be demonstrated.

COMMENT

While the greater magnitude of F over K values on identical filtrates is somewhat disconcerting, the differences encountered were small, as reflected in the conversion factor of 1.04; furthermore, in the case of the urea solutions, the ratio of F to K values was inversed and the differences between them were not statistically significant. Hence, one is justified in concluding that, at least for practical clinical purposes, the F method reliably measures the nonprotein nitrogen content of blood.

The same statement cannot be made for the R method. Not only was there a decided deficiency in nitrogen yields, but this deficiency showed considerable variations and was greatest at the higher nitrogen concentrations. Results obtained from blood filtrates were usually lower than those urea solutions of comparable nitrogen content, a finding which is also inferred in the report of Fee and associates.[4] Hence, our data show not only that the R method fails to quantitatively determine urea, but they also suggest that the method does not measure quantitatively nonprotein nitrogen components, other than urea, which are present in blood.

Peters and Van Slyke[3] pointed out that the hypobromite reaction yielded less than 100 per cent of urea nitrogen. They also reported that ammonia gave off about 95 per cent of its nitrogen, creatinine 14 per cent, uric acid 50 per cent, creatine 67 per cent. Rappaport and Eichhorn[2] admitted that indole compounds, containing nitrogen in their ring system, were not attacked by hypobro-

mite, but they felt that the minimal quantities in which these substances occur could not affect the total nitrogen results. The range of their nitrogen yields from eight blood samples, only two of which represented azotemic levels, was 92 to 105 per cent. Fee and his co-workers[4] found that "urea reacted quantitatively, amino acids gave considerably greater than 100 per cent recovery, and creatinine, uric acid, or adenine much less than theoretical recovery of total nitrogen." Without giving any further details, these authors indicated that the Rappaport and Eichhorn method, when applied to blood filtrates, gave values corresponding to 93 to 94 per cent of the total Kjeldahl nitrogen. These data contrast with our findings of 85 to 97 per cent at nonprotein nitrogen levels of up to 140 mg. per 100 ml. and of 81 to 85 per cent in the higher ranges.

The observed variations in the degree of nitrogen recovery in different blood filtrates must in part be attributed to differences in the proportional representation of individual nonprotein nitrogen components; differences which become more pronounced in the event of nitrogen retention and devolve upon the underlying pathologic process. For example, while the true total azotemia of a patient with hepatic insufficiency might equal that of another patient with primary renal failure, the well-known differences in the respective moieties of urea, amino acids, and other constituents[12] may be expected to cause a considerable disparity in results yielded by the hypobromite method. Inclusion of nonprotein nitrogen fractionation in a study such as ours would be necessary to prove this point. By the same token, much, though not all, of the error inherent in the R method should be expected to be eliminated with the modification of Fee and associates[4] using photometric analysis of the iodine liberated from iodide as a result of the hpobromite reaction: the deficiency in deamination is largely compensated in that colorimeter readings of standard blood filtrates are calibrated against the micro-Kjeldahl values of the same filtrates. On the other hand, calibration by means of standard ammonium sulfate solutions, as recently proposed,[13] should furnish results not much different from those obtained with thiosulfate titration.

Apart from the presumptive lack of quantitative reaction with various nonprotein nitrogen components, the hypobromite reagent manifests a loss in sensitivity at much higher than normal nonprotein nitrogen concentrations. This is inferred by the progressive lowering of recoveries with increasing urea concentrations and, particularly, by the improvement of results when filtrates of azotemic bloods were diluted before analysis. Evidently, as the nonprotein nitrogen approaches the theoretical ceiling of about 300 mg. per 100 ml. of blood,* imposed by the amount of hypobromite used in the procedure, the nitrogen yield becomes increasingly deficient. Consequently, whenever an abnormally high nonprotein nitrogen value is encountered, repeating the procedure on an adequately diluted filtrate will usually reduce the negative error to within 10 per cent.

*This is readily calculated from the formula: $(L-V) \times k = mg.$ NPN per 100 ml., where L and V represent the amounts of thiosulfate solution used in titrating the blank and sample, respectively, and k a factor which equals about 30, varying slightly with the exact thiosulfate titer[2]: with the usual value for L of about 10 ml., a theoretical zero reading for V would give 300 mg. per 100 ml. as the upper limit measurable.

The possibility of analyzing micro-quantities of blood (0.1 ml.) and the elimination of heat-digestion and of a costly photoelectric colorimeter may make the use of the titrimetric method[2] desirable in a small hospital or office laboratory, or for screening purposes. However, it is necessary to keep in mind that it may yield as little as 78 per cent of the true nonprotein nitrogen content.

SUMMARY AND CONCLUSIONS

Rappaport and Eichhorn's hypobromite method for measuring blood nonprotein nitrogen has been compared with the Folin-Wu and micro-Kjeldahl procedures.

The hypobromite method showed a considerable lack of accuracy, since it furnished only 80 to 89 per cent of the Folin values and 78 to 97 per cent of the Kjeldahl values, the lowest yields given by test solutions with the highest nitrogen contents. Dilution of filtrates of azotemic blood samples before subjecting them to the hypobromite reaction approximated the results to the Kjeldahl values. This added step in the procedure is recommended in order to keep the negative error of the method within 10 per cent.

Although the Folin-Wu method tended to give higher results in blood filtrates than the Kjeldahl method, this relationship was reasonably constant throughout the wide range of nitrogen concentrations tested, so that the method can be considered adequate for clinical use. The hypobromite method may, in some respects, seem to be more practical and expedient, but its inherent shortcomings must always be taken into account.

Statistical analyses were carried out by Mr. Myron J. Willis of the Communicable Disease Center.

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A MODIFIED RADIOIODINE TEST FOR THYROID FUNCTION

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SINCE Hertz, Roberts and Evans[1] demonstrated the selective uptake of I^{128} by the thyroid gland, the radioiodine test has become a useful adjunct in the diagnosis of thyroid disease. Although it is probably the most specific indicator of thyroid activity available, there is no general agreement as to the best procedure for differentiation of normal from abnormal thyroid function. The most common methods aim at estimation of the iodide-accumulating ability of the thyroid. This has been used as a reflection of the over-all activity of the untreated gland and correlates well with clinical appraisal of the patient.

The radioiodine-accumulating ability of the thyroid is most often estimated by measurement of the per cent of an initial dose of radioactivity which accumulates in the thyroid gland,[2-4] or is excreted in the urine [5, 6] during a stated time interval. Inherent in both these methods are disadvantages which detract from their clinical usefulness, particularly in cases of borderline and questionable thyroid dysfunction, for the management of which the clinician needs laboratory aids. Since the thyroid must compete with the kidney for the limited quantity of radioiodine in the tracer dose, the amount taken up by the gland in any given period depends not only on its iodine-accumulating ability, but also on renal function. A single in vivo measurement makes no allowance for this important role of the kidney, and may lead to an erroneous diagnosis of thyroid hyper-function in states of renal insufficiency.† When measurements are limited to the total quantity of radioiodine excreted in the urine during a forty-eight or seventy-two hour period, similar errors may occur.[7] Myant and associates[8] have shown that physiological, as well as pathological, alterations of renal function may introduce uncertainty in the determination of thyroid activity by a single measurement, whether it be a direct in vivo count over the gland, or analysis of the total quantity of radioiodine appearing in the urine.

It has been proposed that the iodide-accumulating function of the thyroid be expressed as a rate which can be derived from an analysis of the forty-eight or seventy-two hour cumulative urinary excretion of radioiodine.[11] The calculation of such rates assumes that the disappearance of I^{131} from the blood, its accumulation by the thyroid, and its excretion in the urine are at all times proportional to its concentration in the body fluids, so that these processes may

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†In vivo measurement of radioactivity in the thyroid, although providing the most direct information about the collection of iodine by the gland, also suffers from inaccuracies due to the variation in geometric relationships of the neck structures in different patients, and from the differences in the extent of radiation absorption and backscatter by these tissues.[9, 10] In addition, it necessitates administration of larger doses of radioiodine than do methods utilizing urinary assay alone, so that radiation exposure of the gland is increased.

be represented as simple exponential functions, at least during the first several hours. Later, with the synthesis and release of hormone, the utilization and degradation of the latter by the tissues and the subsequent reappearance of the radioiodine in the blood as inorganic I^- ion, the relationships assume greater complexity and are not adequately described by the original mathematical expressions. Consequently, a method whereby the radioiodine accumulation rate could be obtained from the initial twelve-hour urinary excretion, during which time no significant quantity of labelled thyroid hormone is released,[12] would not only be more convenient to perform, but might be more accurate as well.

This report describes a modified test of thyroid function in which the specific rate constant for I^{131} uptake is determined during the first twelve hours after administration of a tracer dose. The procedure involves multiple determinations of urinary excretion during this period, and the specific accumulation rate by the thyroid gland is then calculated by differential analysis of these data. The results obtained in a group of subjects with normal and pathologic thyroid function are presented.

METHODS

I^{131}, with a half-life of 8.0 days, obtained from Oak Ridge Laboratories of the Atomic Energy Commission as a carrier-free solution of NaI, was assayed with a Radium D+E standard as outlined by the National Bureau of Standards.[13] The usual tracer dose for adults consisted of 10 μc of I^{131}, while proportionately smaller doses on a body weight basis were used for children. The dose was administered in 50 ml. of distilled water to which 10 μg of potassium iodide was added as carrier. The cup was rinsed with another 50 ml. of distilled water containing 10 μg of inert potassium iodide which was also administered to the patient. The total quantity of carrier employed was well below the level at which significant effect on the radioiodide uptake by the thyroid is produced.[14] A solution identical to the administered dose was used as a standard for comparison with urinary and thyroid radioactivities.

The test on adults was usually made on an outpatient basis. The subjects were placed on an iodine-poor diet for a minimum of five days prior to the test, in order to produce a relatively uniform state of "iodine starvation" of their thyroid glands. Persons were excluded who were known to have received organic or inorganic iodine, thyroid extract, or an antithyroid drug within two months prior to the test. The radioiodine solution was administered in the morning to the subjects in the fasting state, in order to promote absorption of the radioiodine. Instructions regarding careful collection of urine specimens were given. If any error in the collection of specimens was discovered, the data were discarded. In the case of infants, whose adequate cooperation for the collection of urine was impossible, a modification of the Hoag apparatus[15] was employed to obtain individual voidings. In most cases the urine was collected at one- to two-hour intervals for the first twelve hours following administration of the radioiodine, and the exact time of each voiding noted. Where longer periods of observation were employed, additional samples were collected and pooled over twelve-hour intervals for thirty-six to sixty hours after the initial collection period. Since the twelve-hour cumulative urinary excretion analysis required the collection of six or more specimens of urine, diuresis was induced in the subject by the ingestion of large quantities of fluid prior to and during the test.

The urine was collected in glass jars to which had been added 1.0 ml. of a stabilizing solution containing sodium hydroxide (0.02M), potassium iodide (0.0015M), and sodium sulfite (0.005M). The total urine volumes were carefully measured and aliquots (usually 1.0 ml.) delivered onto steel planchettes, which were slowly evaporated to dryness under

an infra-red lamp and counted with a thin, end-window Geiger tube mounted in an automatic sample-changing and recording assembly.* All samples were prepared and counted in duplicate, sufficient counts being taken to keep statistical errors within 2 to 5 per cent. These counts were compared with the activity of an aliquot of the standard solution which had been added to a specimen of the subject's control urine, the final volume plated being equal to that of the test platings. Thereby, differences arising from variation in the absorption of the β component of the radiations could be made negligible. The quantity of radioiodine appearing in each urine sample was expressed as a per cent of the administered dose.

When the subject returned with the specimens of urine on the morning following ingestion of the radioiodine, an in vivo measurement of the accumulation by the thyroid gland was made by a modification of the procedure described by Luellen and associates.[16] A bismuth-lined, high gamma efficiency Geiger tube was mounted in an assembly permitting freedom of motion in three dimensions, and shielded by a lead cylinder with a wall 15 mm. in thickness. A 30 mm. square slot in the shield exposed a section along the vertical axis of the tube, which was centered at a point 45 cm. above the patient's thyroid by means of a plumbline. An adjustable neck extension brace was utilized to arch the subject's neck and maintain a relatively constant position with respect to the counter. The shielding principle, as originally described by Seidlin and collaborators[17] was used for these measurements. A solid lead cylinder, 5 cm. long and 5 cm. in diameter which blocked most of the gamma rays from the gland was placed just above the thyroid. Readings taken with this shield in position were subtracted from similar measurements without the shield, thus permitting correction for background radiation; scattered radiation originating from the thyroid and deflected back into the counter from other parts of the body; and direct radiation from I^{131} distributed throughout the rest of the body. A similar set of two readings taken over the midthigh area, permitted some correction for radiation due to radioiodine in the extracellular fluids of the neck. The net thigh reading was subtracted from the net thyroid reading, to yield a value which approximates the radioactivity due to the isotope accumulated in the thyroid gland. This result was then compared with the activity measured at the standard distance above a flask containing the same amount of I^{131} solution as had been administered to the patient, so that the radioiodide accumulation by the thyroid could be expressed as a per cent of the administered dose.

CLINICAL MATERIAL

Radioiodine studies were performed on forty-five subjects, in whom the clinical evidence clearly indicated normal, increased, or decreased thyroid activity. The "normals" comprised twenty-five individuals, twenty-two males and three females, with ages ranging from 5 to 35 years. These subjects presented no evidence of thyroid, intestinal, or kidney disorder. Twelve patients, nine females and three males, ranging in age from 4 to 63 years, had been diagnosed as cases of uncomplicated hyperthyroidism. On the basis of the clinical appraisal and basal metabolism test, five were classified as severe, three as moderate, and four as mild. The hypothyroid group consisted of three males and two females with ages ranging from 2 to 49 years. Two were sporadic cretins, previously untreated. One patient manifested signs and symptoms of hypothyroidism following an acute thyroiditis and another following the removal of a lingual thyroid. The fifth patient was an adult with spontaneous myxedema. In addition, tracer tests were carried out on three patients (ages 6, 11, and 35 years) with renal functional impairment, which in two cases had resulted from chronic nephritis, and in the third from a secondary amyloidosis involving the kidney. Clinically, all three had normal thyroid function.

*Tracerlab, Inc., Boston, Mass.

METHOD OF CALCULATION

The relationship between different indices of thyroid function is clarified by consideration of the dynamics of iodine transport. Fig. 1, as modified from Oddie,[18] describes schematically the pathway of iodine metabolism. Following its administration, iodide ion appears in the blood and body fluids. As soon as it enters the circulation, accumulation by the thyroid and excretion by the kidney begin. The thyroid accumulation rate, K_T, is a measure of the iodine avidity of the gland, and, except in the case of hypothyroidism due to iodine deficiency, reflects the secretory activity of the gland. K_U is a measure of the

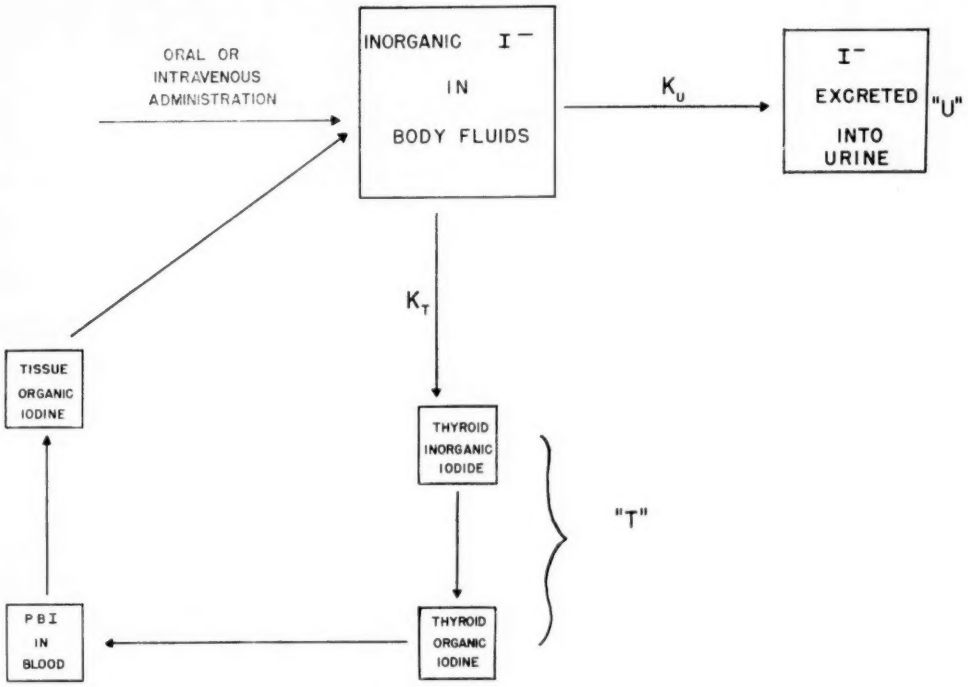

Fig. 1.—Metabolism scheme for iodine.

rate of iodide-elimination by the kidney. The release of I^{131} from the thyroid in the form of labelled hormone is ordinarily slow compared to the initial uptake. Eventually this hormonal iodine is converted to inorganic iodide and reenters the cycle. No other organs of the body have been found to take up significant amounts of iodine[20] and excretion in the feces is ordinarily negligible.[21]

Consider the time course of events when radioiodine has become equilibrated in the body fluids. If U is the total quantity of radioiodine excreted in the urine in time t, C the concentration of the radioiodine in the body fluids, and V the radioiodide space, then the quantity of iodide excreted in the urine in time dt is:

$$\frac{dU}{dt} = K_U C\ V \qquad (1)$$

where K_U is the renal iodide excretion rate. Similarly, if T is the total quantity of radioiodine accumulated by the thyroid gland at time, t, and K_T the thyroid accumulation rate, then the quantity of the radioelement collected in the thyroid gland in time dt is:

$$\frac{dT}{dt} = K_T C\ V \qquad (2)$$

The total loss of isotope from the iodide space is equal to the sum of that excreted in the urine and that collected by the thyroid:

$$V \frac{dC}{dt} = -(K_U + K_T)\ C\ V \qquad (3)$$

Since K_U, K_T and V may be assumed to be relatively constant over short periods of time, the radioiodide concentration falls by a constant proportion of its value during each successive time interval. Integration of equation (3) gives:

$$C = C_o e^{-(K_U + K_T)t} \qquad (4)$$

where C_o represents the initial concentration of the radioiodine if the whole dose were distributed uniformly through the volume represented by the iodide space of the body, V.

Under these conditions, the amount of radioiodine in the thyroid would rise exponentially toward an asymptote, according to

$$T = \frac{K_T C_o V}{K_T + K_U} \left[1 - e^{-(K_U + K_T)t} \right] \qquad (5)$$

and the urinary excretion, U, would likewise rise exponentially, so that:

$$U = \frac{K_U C_o V}{K_T + K_U} \left[1 - e^{-(K_U + K_T)t} \right] \qquad (6)$$

Since the quantity $(C_o V)$ is constant and represents the initial dose of I^{131} administered, D, the terms U and T can be expressed as the proportion of this dose and the equations become:

$$\text{per cent of dose accumulated in thyroid} = 100 \frac{T}{D} = \frac{100\ K_T}{K_U + K_T} \left[1 - e^{-(K_U + K_T)t} \right] \qquad (7)$$

$$\text{per cent of dose excreted} = 100 \frac{U}{D} = \frac{100\ K_U}{K_U + K_T} \left[1 - e^{-(K_U + K_T)t} \right] \qquad (8)$$

As has been shown, a plot of the cumulative urinary excretion of radioiodine over a seventy-two hour period as a function of time, results in a curve the shape of which displays correlation with the states of thyroid activity of the subject.[11] Fig. 2 illustrates such urinary excretion curves from representative hyper-, hypo-, and euthyroid individuals of this study.

These relationships lose their simplicity as time progresses and the processes of synthesis, release of hormone, and tissue utilization become appreciable. Although these events also can be incorporated into a mathematical description, the complexity of the result makes it unsuitable for simple analysis. However, by focusing attention on the initial 12-hour period during which the equations 1 to 8 adequately describe the system, a cumulative urinary excretion curve is obtained which does obey equation (8). The derivative of this curve is:

$$\frac{d(U/D)}{dt} = K_U e^{-(K_U + K_T)t} \qquad (9)$$

Taking the natural logarithm of both sides of the equation yields:

$$\ln \frac{d(U/D)}{dt} = \ln K_U - (K_U + K_T)t \qquad (10)$$

Therefore, if the logarithm of the time derivative of a patient's urinary excretion curve is plotted against the time, a straight line should result. From the slope and the intercept of such a graph, both K_U and K_T can be determined by algebraic manipulation. Such an analysis has been performed on the I^{131} excretion curves of the patients under study. Differentiation of the curves was carried out by the graphical method of Running.[22] Studies on the iodide distribution in normal individuals have revealed that the equilibration of I^- throughout the body fluids is not instantaneous. A rapid increase in V, the "iodide space" occurs during the first hour following the administration of radioiodide, and is followed by a slow increase up to six hours.[8] Because of the lag in the absorption of I^{131} and its diffusion into the body fluids, zero time (t_o) in the present series of experiments has been taken as 1 hour following the administration of the radioiodine.

RESULTS

A. *Comparison of the Performance of Subjects with Normal, Hyper-, and Hypofunction of the Thyroid Gland.*—A typical analysis is illustrated in Fig. 3, which represents a test carried out on a normal patient. The cumulative urinary excretion of radioiodine during the first twelve hours is presented in the top portion, and the lower curve represents the derivative of the upper, plotted on semi-logarithmic paper. It is seen that as the theory predicts, the derivative curve is a straight line.

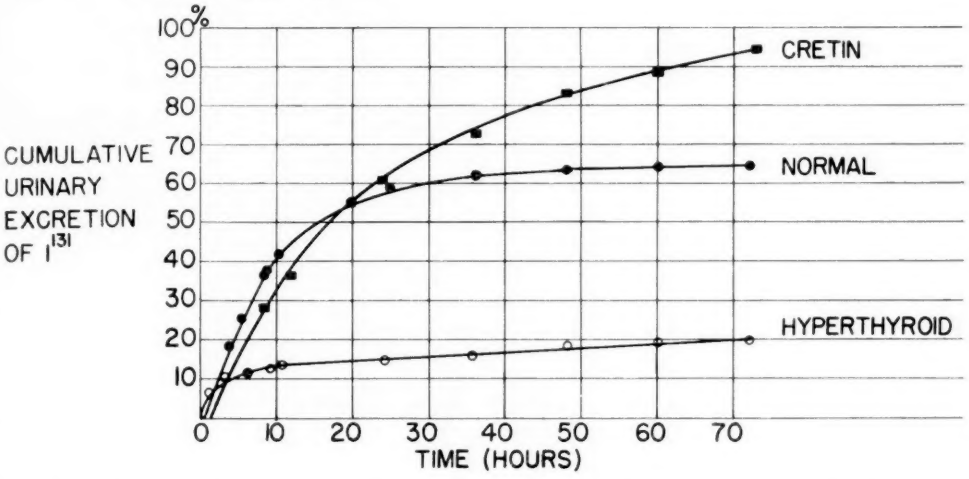

Fig. 2.—Cumulative urinary excretion of I^{131} over a seventy-two hour period for three of the subjects in this study representing the different degrees of thyroid activity. Similar curves have been presented by Keating.[11]

TABLE I. SUMMARY OF RESULTS OF TWELVE-HOUR CUMULATIVE URINARY EXCRETION TESTS

	THYROID ACCUMULATION K_T PER CENT OF REMAINING I ACCUMULATED PER HOUR		RENAL EXCRETION K_U PER CENT OF REMAINING I EXCRETED PER HOUR	
SUBJECTS	MEAN	MAX. RANGE	MEAN	MAX. RANGE
Normal Subjects (25 cases)	5.3 ± 1.7*	(2.0- 9.6)	8.8 ± 1.6*	(6.4-13.0)
Hyperthyroid (12 cases)	25.3 ± 12.6	(12.2-49.3)	8.1 ± 2.4	(4.4-15.0)
Hypothyroid (5 cases)	0.8 ± 0.5	(0- 1.4)	7.8 ± 3.3	(3.4-13.2)

*Standard deviation.

A summary of the results obtained on twenty-five normal, twelve hyperthyroid, and five hypothyroid subjects is presented in Table I. Tests on a number of patients were repeated so that the data represents the results of fifty-one tests in all. It is evident that for this group of patients, the thyroid accumulation rate as determined by the present procedure has furnished a clear separation of the different clinical states of thyroid activity. Although the total number of subjects is small, it seems significant that no overlapping was observed even between the maximum ranges of K_T in the three groups. The mean of the hyperthyroid group is almost five times that of the normal.

which in turn is more than six times that of the clinically hypothyroid group. Also noteworthy is the fact that the renal excretion rate, K_U, as determined by the method outlined is essentially constant in all three groups (Table I), an indication that K_U determined in this manner is free from significant influence by the state of the patient's thyroid function.

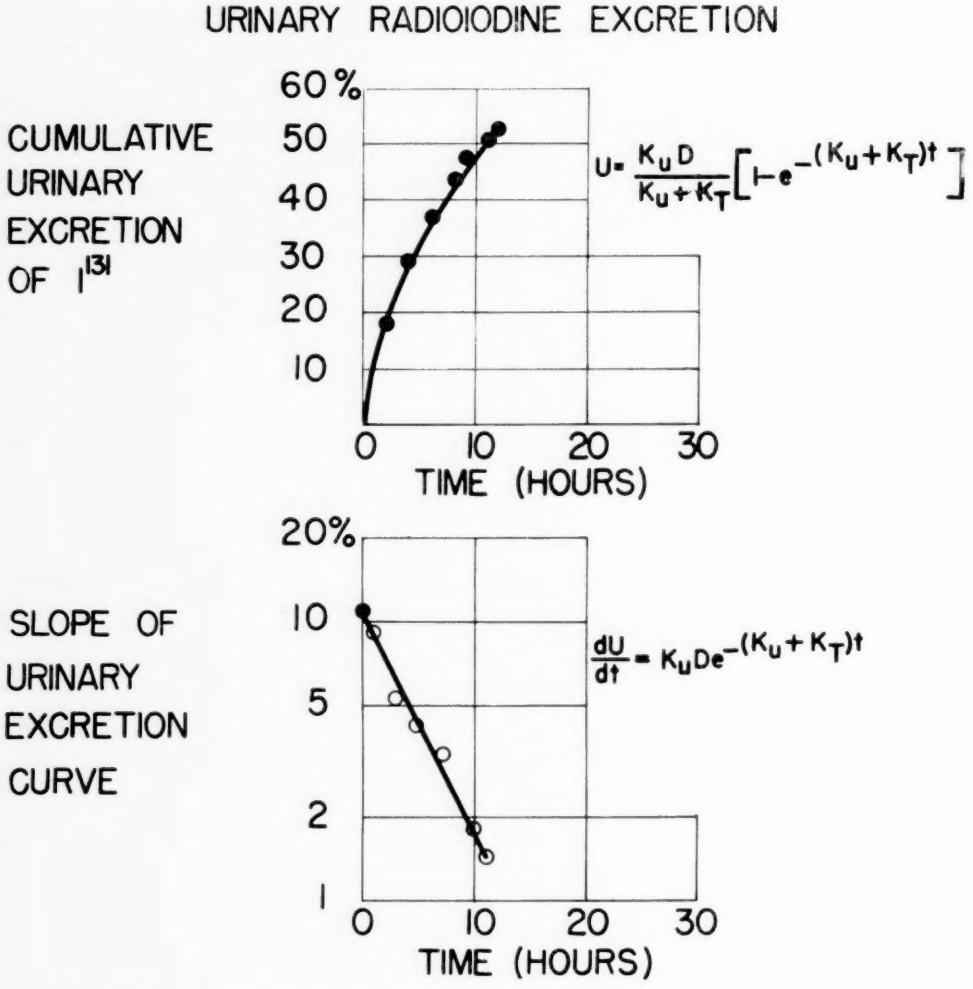

Fig. 3.—The top curve is a plot of the experimental urinary excretion of a normal subject for the first twelve hours following administration of the tracer dose. The lower curve is the derivative of the upper, obtained by graphical differentiation of the original data, plotted on a semi-logarithmic scale (base 10). The points on the derivative curve fall satisfactorily on a straight line as demanded by the theory. The y-intercept of the lower curve at $t=0$ is 11.0 per cent per hour which represents K_U. The logarithmic slope (base e) of the lower graph is 18.1 per cent, which represents the sum of $(K_U + K_T)$. Hence K_T the thyroid accumulation rate is 18.1–11.0 = 7.1 per cent per hour.

As a test of the reproducibility of the method tests were repeated on the same individuals at intervals of one or more weeks. The results of such repeated tests were reproducible well within the range of variation seen for the group as a whole (Table II).

TABLE II. RESULTS OF REPEATED TEST ON SAME PERSONS

SUBJECTS (NORMAL MEN 28-30 YEARS OF AGE)	K_T		K_U	
	1ST TEST	2ND TEST	1ST TEST	2ND TEST
1	6.5	6.1	7.4	9.6
2	5.1	3.3	10.0	7.8
3	8.0	6.8	10.0	16.2
4	5.7	6.8	9.8	13.4
5	3.1	8.4	8.0	10.2
6	6.9	8.6	9.4	9.4
7	6.7	7.1	13.0	11.0

In the calculation of K_T for the hyperthyroids, it was found that the curves for this group of subjects began to deviate earlier than that of the normals from the simple exponential relations of equations 1 to 6. Thus, the urinary excretion of normal subjects usually obeyed a linearly logarithmic curve throughout the first twenty-four hour period after administration of the isotope. In hyperthyroidism the excretion obeyed a simple exponential relationship only during the first twelve hours after which the excretion of I^{131} increased beyond that of the logarithmic law. The direction of the deviation is that to be expected on the basis of an earlier release of labelled hormone by the overactive glands. In the present series of tests it was found that the greater the degree of hyperthyroidism, the more quickly did the phase of simple exponential I^{131} excretion end.

In Table III is presented a summary of the twelve hour total urinary excretion of I^{131} and the twenty-four hour thyroid iodine accumulation as measured by an in vivo count above the gland, in the same three groups of subjects. It is immediately evident that neither of these parameters offers nearly as satisfactory a differentiation of these different thyroid states as does K_T of Table I; and while these particular values represent the performance at the end of only twelve and twenty-four hours, respectively, the results after forty-eight or seventy-two hours also exhibited appreciable overlapping between the three groups of subjects studied. Such overlapping has also been demonstrated by other workers.[11]

B. Cases with Renal Insufficiency.—Table IV summarizes the findings in this group. Three subjects with renal functional impairment, but with clinically normal thyroid function, were tested. In all these patients only a small proportion of the administered dose was excreted in twelve hours. The renal radioiodide excretion values of 1.6 per cent, 3.2 per cent, and 2.3 per cent per hour are

TABLE III. THE TOTAL TWELVE HOUR URINARY EXCRETION AND TWENTY-FOUR HOUR THYROID ACCUMULATION OF I^{131} FOR THE SAME GROUP OF PATIENTS STUDIED IN TABLE I.

SUBJECTS	TOTAL 12-HOUR URINARY EXCRETION OF I^{131}		TOTAL 24-HOUR ACCUMULATION OF I^{131} IN THE THYROID	
	MEAN	RANGE	MEAN	RANGE
Normals	50.2%	38.0-62.5%	35%	18-49%
Hyperthyroids	25.5	7.8-47.0	69	37-90
Hypothyroids	51.6	29.0-70.8	3	0- 7

The thyroid accumulation was measured by the in vivo monitoring procedure described and is much less reliable than the urinary excretion measurement for the small doses of I^{131} employed.

TABLE IV. RESULTS OF TWELVE-HOUR CUMULATIVE URINARY EXCRETION TESTS ON THREE SUBJECTS WITH RENAL INSUFFICIENCY

K_T (PER CENT PER HOUR)	K_U (PER CENT PER HOUR)	12-HOUR I^{131} EXCRETION (PER CENT OF ADM. DOSE)	24-HOUR THYROID I^{131} ACCUMULATION (PER CENT OF ADM. DOSE)
8.8	1.6	10.0	64
8.4	3.2	20.2	79
4.3	2.3	20.6	70

all well below the normal range. However, the thyroid accumulation rates of 8.8 per cent, 8.4 per cent, and 4.3 per cent, respectively, are clearly normal, demonstrating that this method of analysis yields accurate results despite considerable alterations in renal function.

Fig. 4 illustrates the urinary excretion of radioiodine by one of these patients with chronic glomerulonephritis and renal failure (impaired concentrating ability, NPN retention, depressed urea clearance). In vivo monitoring of the thyroid gland at the end of a 72-hour period revealed an uptake of 70 per cent of the administered dose. Analysis of the urinary radioiodine elimination confirmed this figure, since 33 per cent of the administered dose was excreted during that time. On the basis of these data alone, the existence of overactivity of the thyroid gland in this patient might have been inferred. However, the thyroid iodide accumulation rate was 4.3 per cent per hour, a value well within normal limits.

C. Effect of Inert Iodide.—One of the important deterrents to the more widespread application of the radioiodine tracer test for the diagnosis of thyroid dysfunction has been the fear of radiation hazard to the thyroid gland as a result of the selective concentration of the isotope in this organ. If 50 per cent of a 10 μc dose is taken up and uniformly distributed through a thyroid weighing 25 Gm., the thyroid will receive a total radiation dose of about 12 r., while dosage to other parts of the body will not exceed the recommended limits of tolerance of 0.3 r. per week.[23] It is obvious that by reducing the size of the tracer dose the effects of the irradiation are diminished. Quantities of I^{131} as small as 1.5 μc have sufficed to carry out the twelve-hour urinary excretion analysis in infants without compromising the accuracy of the procedure. In addition, however, it has been found that by administration of potassium iodide at the end of the twelve-hour period further accumulation of the isotope can be inhibited so that irradiation of the gland is reduced even more.

In eight normal individuals the I^{131} tracer test was carried out with serial measurement of the activity over the thyroid gland during the interval between twelve to seventy-two hours following administration of the isotope. At the termination of the twelve-hour urine collection period, KI was orally administered and its effect upon the in vivo collection of the radioiodine noted. In all cases the administration of 1-1.5 Gm. of KI stopped the uptake of I^{131} immediately. Without such treatment, the accumulation continues, reaching a maximum at approximately twenty-four hours.[16] Hence, the amount of radioactivity stored in the gland can be materially reduced by this procedure. The effect of 1 Gm. of KSCN was qualitatively similar to the results obtained with KI. In approximately half of the patients, the administration of several doses

of KI not only arrested the uptake of I^{131} by the gland, but also induced a more rapid loss of radioiodine from the gland. Fig. 5 presents a typical result of this kind.

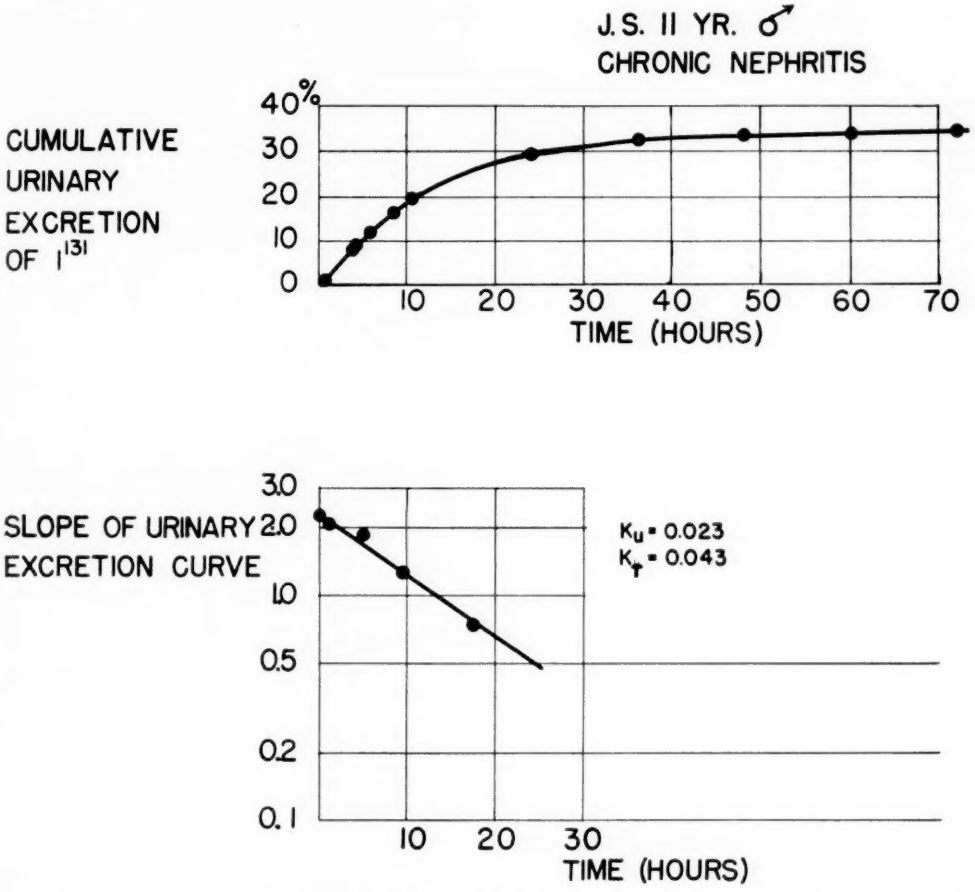

Fig. 4.—I^{131} excretion curve (top) and its time-derivative (lower curve) for a subject with a normal thyroid but impaired renal function.

DISCUSSION

The procedure here described permits a shorter urine collection period than has hitherto been employed, without loss of accuracy in evaluation of the iodine avidity of the gland. The convenience of the procedure permits its application to routine clinical use.

From the twelve-hour urinary excretion data, estimations of the asymptotic value of the urinary excretion can be made (renal fraction of Keating and associates[11]). From equation (6) it can be seen that, as t gets larger, U/D the fraction of radioiodine excreted in the urine, approaches a limiting value, U/D of:

$$\lim_{t \to \infty} U/D = U\infty/D = \frac{K_U}{K_U + K_T} \quad (9)$$

In Table V, the values of $U\infty$ calculated by this equation from the twelve-hour

test are presented and compared with the actual seventy-two hour urinary excretion, which was experimentally determined in a group of thirteen normal individuals. It is evident that agreement is close. There is a small but systematic deviation in the direction of the observed excretion exceeding that calculated on the basis of the simple analysis. This trend probably represents a small release of labelled hormone by the gland.

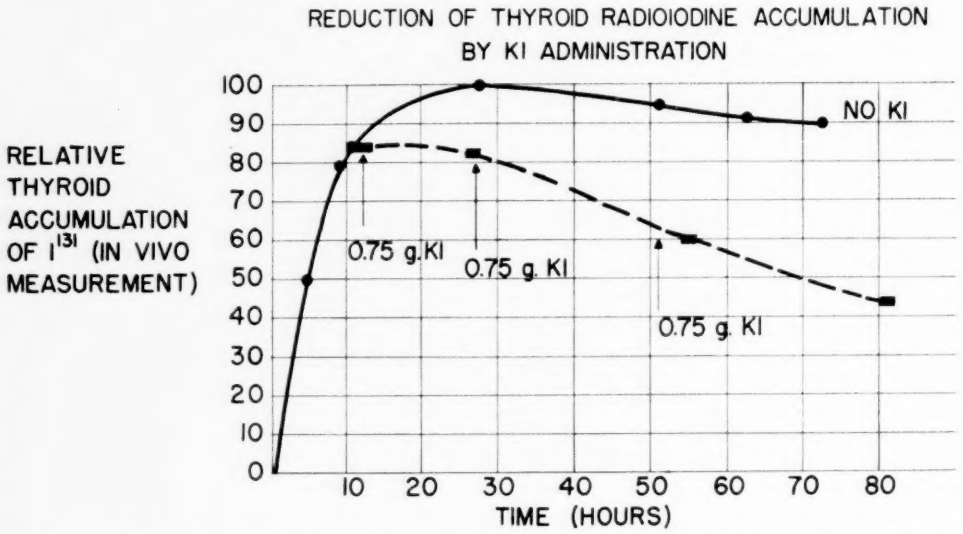

Fig. 5.—Two tests on the same individual showing the effect on the total I^{131} accumulation in the thyroid, of the administration of KI after the end of the twelve-hour urine collection period.

TABLE V. COMPARISON OF ESTIMATED AND OBSERVED ASYMPTOTIC VALUE OF THE URINARY EXCRETION ($U\infty$) IN THIRTEEN NORMAL SUBJECTS

PATIENT	ESTIMATED $U\infty$	OBSERVED $U\infty$ (AFTER 72 HOURS)
1	48.8	47.5
2	52.1	65.5
3	54.0	59.0
4	68.5	69.5
5	55.0	68.5
6	55.0	56.5
7	52.0	55.5
8	73.3	79.0
9	75.4	81.0
10	74.0	81.0
11	70.3	64.5
12	59.5	65.0
13	57.0	64.0

The collection of a seventy-two hour urine sample does have the advantage of affording a check on the total urinary excretion of the isotope. However, the difficulty and inconvenience of obtaining a complete seventy-two hour urine collection militates against its routine use. An in vivo measurement of the total thyroidal I^{131} accumulation, though less precise with small doses of the isotope, is sufficiently accurate to serve as a check against the possibility of incomplete urine collection.

That the radioiodine accumulation by the thyroid can be more precisely and quantitatively evaluated by expressing it as a rate, rather than as the total urinary excretion or thyroidal collection of I^{131}, has been borne out in the present study. The large overlap in the twelve-hour urinary excretions, and in the twenty-four hour thyroidal accumulations of the radioelement, between individuals with normal and abnormal thyroid function does not occur when K_T, the thyroid accumulation rate, is used as the discriminating parameter. The thyroid radioiodine accumulation rate clearly separated the subjects in which normal, hyper- or hypothyroidism was established on the basis of clinical and laboratory evidence. In the case of altered iodine-accumulation associated with impaired renal function, such a rate analysis is the only radioiodine procedure which can reliably determine the state of thyroid function.

With equipment now available, a twelve-hour cumulative urinary excretion analysis can be readily carried out in children with doses as small as 1 to 2 μc of I^{131} (and even less if a scintillation counter is available). When doses of this magnitude are employed and KI administered after twelve hours to prevent further radioisotope uptake, the total irradiation of the thyroid is reduced to 5 to 6 r, or less, an amount comparable to that received by the entire chest in standard fluoroscopic procedures. The method described here would appear to be applicable to the routine investigation of thyroid function in infants and children.

The twelve-hour I^{131} excretion values obtained here are generally somewhat higher than those observed in other studies.[6, 11] This may be accounted for by the enhanced excretion of the radioelement under the conditions of diuresis employed. From the limited observations obtained in this study, there appears to be a correlation of the renal excretion of the radioelement with the rate of urine flow.

SUMMARY

A method is described whereby the specific rate constant for I^{131} uptake by the thyroid can be determined from measurements of the urinary excretion of I^{131} during the first twelve hours after administration of the tracer dose. The values obtained are quantitatively reproducible, subject to less error than measurements over the gland and the procedure is sufficiently convenient to permit its routine clinical use. Results of the tests in groups of euthyroid, hyperthyroid, and hypothyroid individuals have shown good correlation with clinical estimates of thyroid activity. The advantage of such a test in yielding a suitable parameter of thyroid function in the face of physiologic or pathologic alterations of renal function are presented. By the administration of inert iodide after a relatively short period of urine collection, it is possible to decrease substantially the amount of irradiation which the gland would otherwise receive from the radioisotope.

We are indebted to Dr. Harry H. Gordon for his extremely helpful suggestions and criticisms.

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